REMARKS

Claims 1-114 are pending. Claims 6-10, 19, and 46-114 have been withdrawn from consideration.

Claims 1-5, 11-18, and 20-45 stand rejected.

None of the above changes raise any issue of patentability. Both before and after the above changes, the invention was described in full, clear, concise, and exact terms and met all conditions for patentability under 35 USC 101 *et seq*. The scope of the claims of any resulting patent (and any and all limitations in any of said claims) shall not under any circumstances be limited to their literal terms, but are intended to embrace all equivalents. Accordingly, under no circumstances whatsoever may these claims be interpreted as:

1. having been altered in any way for any reason related to patentability;

2. having been narrowed;

3. a concession that the invention as patented does not reach as far as the original, unamended claim;

4. a surrender of any subject matter as a condition of receiving a patent; and/or,

5. estopping applicants from asserting infringement against every equivalent, whether now known or later developed, foreseen or unforeseen.

Applicants also emphasize that the decision to address the Examiner's suggestions via claim amendment with the understandings set forth above is not in any way intended to avoid the "gatekeeping" role of the PTO with regard to the examination and issuance of valid patents for patentable inventions.

I. 35 U.S.C. § 112 FIRST PARAGRAPH REJECTIONS

The Examiner has rejected claims 1-5, 11-18, and 20-45 as not enabled. The Examiner states:

> while being enabling for the particular compounds having the particular formula herein as FBPase inhibitor in combination with glyburide and other particular agents as insulin secretagogue, employed in composition herein, does not reasonably provide enablement for co-administering any

compounds represented by a FBPase inhibitor and an insulin secretagogue recited in the claims herein. (Office Action pp. 2-3)

The Examiner cites *In re Wands* and then sets out the factors:

The nature of the invention: The instant invention pertains to a pharmaceutical composition for treating diabetes in a mammal.

The relative skill of those in the art: The relative skill of those in the art is high.

The breadth of the claims: The instant claims are deemed very broad since the instant claims read on any compounds represented by a FBPase inhibitor and an insulin secretagogue employed in the composition herein.

The amount of direction or guidance presented: Functional language at the point of novelty, as herein employed by Applicants, is admonished in *University of California v. Eli Lilly and Co.* 43 USPQ2d 1398 (CAFC 1997) at 1406; stating this usage does "little more than outline goal appellants hope the recited invention achieves and the problems the invention will hopefully ameliorate". The CAFC further clearly states that "[A] written description of an invention involving a chemical genus, like a description of a chemical species, requires a precise definition, such as structure as by structure, formula, [or] chemical name, of the claimed subject matter sufficient to distinguish it from other materials" at 1405(emphasis added), and that "it does not define any structural features commonly possessed by members of the genus that distinguish from others. One skilled in the art there cannot, as one can do with a fully described genus, visualize or recognize the identity of the members of the genus. A definition by function, as we have previously indicated, does not suffice to define the genus.." at 1406 (emphasis added).

In the instant case, 'represented by a FBPase inhibitor" and "an insulin secretagogue", recited in the instant claims are purely functional distinction. Hence, these functional recitations read on any compounds that might have the recited functions. However, the specification merely provides those particular compounds for each kind of functional compounds for the composition.

Thus Applicants functional language at the points of novelty fails to meet the requirements set forth under 35 U.S.C. 112, first paragraph. Claims employing functional language at the exact point of novelty, such as Applicants', neither provide those elements required to practice the inventions, nor "inform the public during the life of the patent of the limited of monopoly asserted" (citation omitted)

The predictability or unpredictability: The instant claimed invention is highly *unpredictable* as discussed below:

It is noted that the pharmaceutical art is unpredictable, requiring each embodiment to be individually assessed for physiological activity. *In re Fisher*, 427 F.2d 833, 166 USPQ 18 (CCPA 1970) indicated that the more unpredictable an area is, the more specific enablement is necessary in order to satisfy the statute. In the instant case, the instant claimed invention is highly unpredictable since one skilled in the art cannot fully described genus, visualize or recognize the identity of the members of the genus, by structure, formula, or chemical name, of the claimed subject matter, as discussed above in *University of California v. Eli Lilly and Co.* Hence, in the absence of fully recognizing the identity of the members genus herein, one of skill in the art would be unable to fully predict possible physiological activities of any compounds having claimed functional properties in the pharmaceutical compositions herein.

Moreover, one of skill in the art would recognize that it is highly unpredictable in regard to therapeutic effects, side effects, and especially serious toxicity that may be generated by drug-drug interactions when and/or after administering to a mammal, the **combination** of any compounds represented by a FBPase inhibitor and an insulin secretagogue, which may encompass more than a thousand compounds. See text book "Goodman & Gilman's The Pharmacological Basis of Therapeutics" regarding possible drug-drug interactions (9th ed, 1996) page 51 in particular. This book teaches that "The frequency of significant beneficial or adverse drug interactions is unknown" (see the bottom left column of page 51) and that "Recognition of beneficial effects and recognition of and prevention of adverse drug interactions require a thorough knowledge of the intended and possible effects of drugs that are prescribed" and that "The most important adverse drug-drug interactions occur with drugs that have serious toxicity and a low therapeutic index, such that relatively small changes in drug level can have significant adverse consequences" (see the right column of page 51) (emphases added). In the instant case, in the absence of fully recognizing the identity of the members genus herein, one of skill in the art would not be able to fully predict possible adverse drug-drug interactions occurring with many combinations of any compounds having claimed functional properties in the pharmaceutical compositions herein to be administered to a host. Thus, the teachings of the book clearly support that the instant claimed invention is highly unpredictable.

The presence or absence of working examples and the quantity of experimentation necessary: As discussed above, only those particular

compounds for each kind of functional compounds employed in the composition herein is disclosed in the specification. It is noted that only one particular combination of Compound J and glyburide, was tested and is shown in Example X at page 315-316 of the specification. Thus, the evidence in the examples is also not commensurate in scope with the claimed invention and does not demonstrate criticality of a claimed range of the active agents or compounds in the claimed composition. See MPEP § 716.02(d)

Thus, the specification fails to provide sufficient support of the broad use of any compounds having those functions recited in the instant claims. As a result, necessitating one of skill to perform an exhaustive search for the embodiments of any compounds having those functions recited in the instant claims suitable to practice the claimed invention.

Genentech, 108 F.3d at 1366, states that "a patent is not a hunting license. It is not a reward for search, but compensation for its successful conclusion" and "[p]atent protection is granted in return for an enabling disclosure of an invention, not for vague intimations of general ideas that may or may not be workable".

Therefore, in view of the Wands factors, the case University of California v. Eli Lilly and Co. (CAFC 1997) and In re Fisher (CCPA 1970) discussed above, to practice the claimed invention herein, a person of skill in the art would have to engage in undue experimentation to test all compounds encompassed in the instant claims and their combinations employed in the claimed compositions to be administered to a host, with no assurance of success. (Office Action pp. 2-7)

The Examiner then addresses the Applicants' arguments saying:

It is the examiner's position that Applicants functional language at the points of novelty fails to meet the requirements set forth under 35 U.S.C. 112, first paragraph. Claims employing functional language at the exact point of novelty, such as Applicants', neither provide those elements required to practice the inventions, nor "inform the public during the life of the patent of the limited monopoly asserted"...as pointed out in the previous Office Action.

Applicant argues that the novelty of the invention is not either an FBPase inhibitor or an insulin secretagogue, but the point of novelty is the combination of these two agents. Applicant's arguments are not found persuasive and convincing since the point of novelty is the combination of these two agents, **both represented by functional languages**. Hence, the

functional languages in the claims are employed as the essential and critical elements of the claimed invention.

Claims are given their **broadest** reasonable interpretation. In this case, the instant claims are **not** limited to those particular compounds having the particular formula herein as FBPase inhibitor in combination with glyburide and other particular agents as insulin secretagogue in the specification. On the contrary, the instant claims read on administering to a patient the **combination** of any compounds represented by FBPase inhibitor and an insulin secretagogue.

"Applicants believe that a person of ordinary skill in the art could determine which compounds are FBPase inhibitors through the use of routine experimentation, such as described in Examples A and B". Applicant also asserts that "With the use of high-throughput screening, there is nothing undue about the amount of experimentation needed to determine what compounds are encompassed by the claims".

Contrary to Applicant's assertion, these functional recitations may reasonably encompass those known and **unknown** or **future known** compounds having the recited functions as of the instant filing date. Note that those **future known** compounds have not yet been discovered and/or made as of the filing date. Hence, those unknown or future compounds encompassed by claim 1 **must** require to additional or future research to discover, establish, make and/or verify their usefulness, for example, including "with the use of high-throughput screening". Therefore, as indicated in the previous Office Action, the skilled artisan has to exercise **undue experimentation** to practice the instant invention.

As pointed out in the Previous Office Action, *Genentech*...states that "a patent is not a hunting license. It is not a reward for search, but compensation for its successful conclusion" and "[p]atent protection is granted in return for an enabling disclosure of an invention, not for vague intimations of general ideas that may or may not be workable" (emphasis added).

Note that Applicant own research paper is seen to support the examiner position herein, undue experimentation involved herein (see Erion et al. Book of Abstracts, 219th ACS National Meeting, San Francisco, CA, March 26-30, 2000(2000), COMP-029. American Chemical Society: Washington, D.C.):

AB "Drug discovery targeting nucleotide binding sites have largely failed due to difficulties in finding ligands with high binding affinities, good enzyme specificities, and suitable cell penetration properties. The poor

success is attributed primarily to two reasons. First, ligands with high binding affinities usually require a neg. charged group and therefore have limited cell penetration. Second, ligands with high enzyme specificities are rare and difficult to design due to the structural similariy of nucleotide binding sites and their high abundance in nature. In an effort to bypass these challenging design hurdles and identify nucleotide mimetics that bind to the AMP site of fructose 1,6-bisphosphatase, we calcd. the relative binding free energies for 15 AMP analogs using free energy perturbation methodol. and the human FBPase-AMP structure. Calcd. binding free energy differences were in good agreement with the relative inhibitory potencies detd. eptl. The results of the study suggest that free energy calcns. are useful for characterizing nucleotide binding sites and identifying interactions that are essential for high binding affinity and specificity". (emphasis added)

Thus, Applicants clearly acknowledge and understand the difficulties and poor success in finding suitable drug candidates. Let alone the **combination** of any compounds represented by an FBPase inhibitor and an insulin secretagogue, encompassed by the claims, i.e., those known and **unknown** or **future known** compounds.

It is noted that only one particular combination of Compound J and glyburide, was tested and is shown in Example X at page 315-316 of the specification.

Thus, the specification fails to provide clear and convincing evidence in sufficient support of the broad use of any compounds having those functions recited in the instant claims. As a result, necessitating one of skill to perform an exhaustive search and undue experimentation for the embodiments of any compounds having those functions recited in the instant claims suitable to practice the claimed invention.

As discussed in the previous Office Action, one of skill in the art would recognize that it is highly unpredictable in regard to therapeutic effects, side effects, and especially serious toxicity that may be generated by drug-drug interactions when and/or after administering to a host (i.e., a mammal) the **combination** of any compound represented by the functional languages...In the absence of fully recognizing the identity of the members genus herein, one of skill in the art would not be able to fully predict possible adverse drug-drug interactions occurring with many combinations of any compounds having claimed functional property herein to be administered to a host. Thus, the teachings of the book clearly support that the instant claimed invention is highly unpredictable. (Office Action pp.8-11)(internal citations omitted)

The Applicants respectfully traverse this rejection.

The Examiner finds that the functional language occurs at the point of novelty (Office Action p. 7). However, the novelty of the invention is not either an FBPase inhibitor or an insulin secretagogue, but the point of novelty is the combination of these two agents.

The Examiner objects to the use of a functional definition, in spite of case law to the contrary. As stated in MPEP § 2173.05(g), there is nothing inherently wrong with defining some part of an invention through functional terms. In fact the use of functional language has explicitly been approved by the Court of Appeals. When discussing functional language in *Swinehart*, the Court said:

> In our view, there is nothing intrinsically wrong with the use of such a technique in drafting patent claims. Indeed, we have even recognized in the past the practical *necessity* for the use of functional language. *In re Swinehart and Sfiligoj*, 169 U.S.P.Q. 226, 228 (C.C.P.A. 1971).

Furthermore, MPEP § 2173.01 states:

> Applicants may use functional language, alternative expressions, negative limitations, or any style of expression or format of claims which makes clear the boundaries of the subject matter for which the protection is sought. As noted in *In re Swinehart*, 439 F.2d 210, 160 USPQ 226 (CCPA 1971), a claim may not be rejected solely because of the type of language used to define the subject matter for which patent protection is sought.

For example, *In re Barr*, the U.S. Court of Customs and Patent Appeals approved the use of functional language in defining the term "incapable of forming a dye with said oxidized developing agent." *See In re Barr*, 170 U.S.P.Q. 330, 337 (C.C.P.A. 1971). The Court went on to say that:

> In summary, we hold that an applicant may invoke the third paragraph of section 112 to justify the specification of one or more elements of a claimed compound in "functional" terms, and that those "functional" terms may be "negative." The real issue in any such case is not whether the recital is "functional" or "negative," but whether the recital sets definite boundaries on the patent protection sought - that is, whether those skilled in the relevant art can determine what the claim does or does not read on. Judged by this standard, we think it clear that the controverted language complies with the second paragraph of section 112. *Id.*

Furthermore, a "limited use of terms of effect or result, which accurately define the essential qualities of a product to one skilled in the art, may in some instances be permissible and even desirable."

In re Fuetterer, 138 USPQ 217, 222 (C.C.P.A. 1963)(quoting *General Electric Co. v. Wabash Appliance Corp.*, 37 USPQ 466, 469 (U.S. 1938)).

The present situation is similar to the *In re Fuetterer* case. In that case, the examiner and the Board rejected certain composition claims as indefinite, ambiguous, unduly broad, and functional, in part because the term "inorganic salts" was defined in a functional way. *Id.* at 218-219. The examiner stated that:

> "Inorganic salt" reads on literally thousands of materials, many of which would *not be operative* for applicant's purpose. For example, some salts *could* readily react with other ingredients in the composition while other salts *could* be corrosive or destructive of the rubber. This recitation is functional since it merely describes how the salt functions as the surface of the tire wears away. *Id.* at 220.

First, the Court found that use of functional language was proper. *Id.* at 222. Then the Court went on to say that the claims were not unduly broad. *Id.* at 223. The Court stated:

> in the words of the *second* paragraph of section 112, "applicant regards as his invention" the combination with his other tread ingredients of *any* inorganic salt *capable* of "maintaining the carbohydrate, the protein, or mixture thereof, in colloidal suspension* * *." It is exactly this combination which appellant has particularly pointed out and *distinctly claimed* in compliance with the *second* paragraph of section 112...Appellant's invention is the *combination* claimed and not the discovery that certain inorganic salts have colloidal suspending properties. We see nothing in the patent law which requires appellant to discover which of all those salts have such properties and which will function in combination. *Id.*

The Court went on to point out that there was no "undue burden" caused by the functional language of the claims:

> The Patent Office would require him to do research on the "literally thousands" of inorganic salts and determine which of these are suitable for incorporation into his claimed combination, apparently forgetting that he has not invented and is not claiming colloidal suspending agents but tire stock composed of a combination of rubber and other ingredients. *Id.*

In addition, numerous other cases have found that the use of functional language is acceptable. *See e.g. In re Herschler*, 200 U.S.P.Q. 711, 717 (C.C.P.A. 1979)(disagreeing with the solicitor who said

that a single example of a steroid in the specification could not describe the class of steroids claimed in a functional manner); *In re Edwards*, 196 U.S.P.Q. 465, 467 (C.C.P.A. 1978)(stating that the application is "not intrinsically defective merely because appellants chose to describe their claimed compound by the process of making it); *In re Mattison*, 184 U.S.P.Q. 484, 486 (C.C.P.A. 1975)(saying "General guidelines are disclosed for a proper choice of the substituent Ep together with a representative number of examples."); *Ex parte Schundehutte*, 184 U.S.P.Q. 697 (Bd. Pat. App. & Int. 1974).

In particular, examples of the claimed compounds are often found to be sufficient to guide a person of ordinary skill in the art when it comes to functional claims. For instance, in the *Ex parte Schundehutte* case, the Examiner rejected a claim which read "A reactive dyestuff of the formula...is the radical of an organic dyestuff in which –N-R'_1 is bonded directly to an aromatic nuclear carbon atom of F..." *Ex parte Schundehutte*, 184 U.S.P.Q. at 697. The examiner also found the claim was not enabled and lacked written description, because "examples and other exemplary material in the disclosure is not adequately representative of the area covered by the claims and does not provide 'assurance that all of the compounds falling within the scope of the claims will dye fabrics with asserted properties.'" *Id.* at 698. The Board did not agree that the claims were indefinite saying

> While specific methods of use and/or dyeing properties for each and every species covered by the claims have not been demonstrated as pointed out by the examiner, a disclosure of that extent is not required by statute...In the present case, we believe that those skilled in the art could effectively use the reactive dyestuff compounds of the scope covered by the claims, at least without undue experimentation, from the present written description of the invention in the specification, including the numerous examples therein, and from the art recognized properties of dyestuff compounds and conventional methods of using such compounds which those skilled in the dyestuff art are presumed to know. *Id.*

Moreover, the Examiner's legal position that functional language may not be used at the point of novelty is directly contrary to established Federal Circuit law. As the C.C.P.A. said in *In re Swinehart and Sfiligoj*:

> Our study of these cases...satisfies us that any concern over the use of functional language at the so-called 'point of novelty' stems largely from the fear that an applicant will attempt to distinguish over a reference disclosure by emphasizing a property or function which may not be mentioned by the reference and thereby assert that this claimed subject matter is novel. Such a concern is not only irrelevant, it is

> misplaced...[W]here the Patent Office has reason to believe that a functional limitation asserted to be critical for establishing novelty in the claimed subject matter may, in fact, be an inherent characteristic of the prior art, it possesses the authority to require the applicant to prove the subject matter shown to be in the prior art does not possess the characteristics relied on. *In re Swinehart and Sfiligoj*, 169 USPQ 226, 228-29 (C.C.P.A. 1971).

Likewise, in *Ex parte Skinner*, the Board of Patent Appeals and Interferences also acknowledged that a functional limitation may provide novelty to a claim that is otherwise anticipated by a reference. *Ex parte Skinner*, 2 USPQ2d 1788, 1789 (Bd. Pat. App. Int'f 1986). In *Skinner*, the examiner had rejected as anticipated a claim to a mold for making contacting lenses on the basis that the only limitations of the claim not explicitly disclosed by the cited prior art were characteristics of the mold, and that such characteristics may have been inherently present in the prior art mold. *Id.* at 1788. The Board rejected the Examiner's reasoning and explained that the examiner had failed even to make out a *prima facie* case of anticipation:

> We are mindful that there is a line of cases represented by *In re Swinehart*, 439 F.2d 210, 169 USPQ 226 (CCPA 1971) which indicates that where an examiner has reason to believe that a functional limitation asserted to be critical for establishing novelty in the claimed subject matter may, in fact, be an inherent characteristic of the prior art, the examiner possesses the authority to require an applicant to prove that the subject matter shown to be in the prior art does not possess the characteristic relied on. Nevertheless, before an applicant can be put to this burdensome task, the examiner must provide some evidence or scientific reasoning to establish the reasonableness of the examiner's belief that the functional limitation is an inherent characteristic of the prior art. *Id.* at 1789.

The Board explained that "[a]bsent reasons on the part of the examiner regarding why the natural result of the process used to prepare the mold of [the prior art reference] would have been to achieve the characteristics claimed by appellant's mold, a *prima facie* case of anticipation has not been established." *Id.*

The case law is clear that functional language may provide novelty to a claim that is otherwise anticipated by a prior art reference. *See In re Ludtke*, 441 F.2d 660, 664, 169 USPQ 563 (C.C.P.A. 1971) ("[s]ince the only alleged distinction between claims 1-6 and [the cited reference] is functional language, it was incumbent upon appellants, when challenged, to show that the canopy disclosed by [the

reference] does not actually possess such characteristics."); *See also In re Mills*, 916 F.2d 680, 682-83, 16 USPQ2d 1430, 1432-33 (Fed. Cir. 1990) ("*Ludtke* ... dealt with a rejection for lack of novelty, in which case it was proper to require that a prior art reference cited as anticipating a claimed invention be shown to lack the characteristics of the claimed invention .... *That proof would in fact negate the assertion that the claimed invention was described in the prior art.*") (citing *In re Ludtke*, 441 F.2d 660, 169 USPQ 563 (CCPA 1971) (emphasis added).

The term "M" is defined functionally in claim 11 in that "wherein *in vivo* or *in vitro* compounds of formulae I and IA are converted to M-PO_3^{2-}, which inhibits FBPase." In other words, M must be a group that *in vivo* exists as M-PO_3^{2-} and is an inhibitor of FBPase. Additionally, the specification at p. 25, lines 21-28 explains what is meant by an FBPase inhibitor.

> Combinations of the invention include at least one FBPase inhibitor. In most embodiments, the combination will include one FBPase inhibitor. FBPase inhibitors used in the invention are compounds that can inhibit human FBPase activity (Examples A-B), inhibit glucose production from hepatocytes (Examples C-D), lower glucose levels in fasted animals (Examples E-G), or decrease blood glucose levels in diabetic animal models (Examples V and W). Preferred FBPase inhibitors are compounds that inhibit enzyme activity as determined by conducting *in vitro* inhibition studies (Examples A and B).

Like the applicant *In re Fuetterer*, the Applicants are not claiming insulin secretagogues and FBPase inhibitors, but instead a composition containing insulin secretagogues and FBPase inhibitors. Consequently, the Applicants need not list the name or structure of every compound capable of acting as an insulin secretagogue or an FBPase inhibitor.

Furthermore, the Applicants believe that a person of ordinary skill in the art could determine which compounds are FBPase inhibitors through the use of routine experimentation, such as described in Examples A and B. With the use of high-throughput screening, there is nothing undue about the amount of experimentation needed to determine what compounds are encompassed by the claims.

The specification also names several known insulin secretagogues and explains how to determine if other compounds are insulin secretagogues:

> Insulin secretagogues used in this invention typically exhibit activity in assays known to be useful for characterizing compounds that act as insulin secretagogues. The assays include, but are not limited to, those

identifying the following exemplified activities: (a) insulin release from pancreatic islets or beta cell lines (Example H), (b) insulin secretion a rat (Example L), (c) glucose lowering in a fasted rat (Example I), (d) intravenous or oral glucose tolerance in a fasted rat (Examples J and K), (e) inhibition of ATP-dependent potassium channels in pancreatic beta cells (Example M), (f) binding to the sulfonylurea receptor (Example N), (g) binding to the GLP-1 receptor, and (h) inhibition of DPP-IV (Example O). Further assays include those described in Bergsten P et al. J. Biol. Chem. 269: 1041-45 (1994); Frodin M et al J. Biol. Chem. 270: 7882-89(1995); Dickinson K et al Eur. J. Pharmacol. 339: 69-76 (1997); Ladriere L et al. Eur. J. Pharmacol. 335: 227-234 (1997); Edwards G, Weston AH Ann. Rev. Pharmacol. Toxicol. 33: 597-637 (1993); Aguilar-Bryan L. et al. Science 268: 423-6 (1995); Thorens B et al. Diabetes 42: 1678-82 (1993); Deacon CF, Hughes TE, Holst JJ Diabetes 47: 764-9 (1998). Especially preferred insulin secretagogues are glyburide, glipizide, and glimepiride, mitiglinide, BTS-67582, repaglinide, and nateglinide. (p. 206, lines 8-23)

The Examiner concludes that the pharmaceutical arts and this invention are unpredictable. (Office Action pp. 7-8) In Example X, the Applicants have shown that the use of this invention results in improved glycemic control. The Applicants are not sure why the Examiner believes that a person of ordinary skill in the art would need to be able to fully predict all possible adverse drug interactions, in order for this invention to be fully enabled. This is not a requirement of section 112.

The Examiner points to Example X as a working example and says that the specification does not provide support for the broad claims. (Office Action pp. 8-9) The Applicants believe that a person of ordinary skill in the art could use the entire scope of the claimed invention without undue experimentation. As explained above, a person of ordinary skill in the art can easily identify what compounds fall within the scope of the claims.

The Examiner maintains that determining which compounds meet the requirements of the claims will require undue experimentation. However, the routine nature of High Throughput Screening is evidenced by many pre-July 2000 articles including Burbaum and Sigal, New Technologies for High-Throughput Screening, *Curr. Opin. Chem. Biol.* **1997**, **1**:72-78; Auer, *et al.*, Fluorescence Correlation Spectroscopy:Lead Discovery by Miniaturized HTS, *Drug Discovery Today* **1998**, **3**(10):457-465; and

Silverman, *et al.*, New Assay Technologies for High-Throughput Screening, *Curr. Opin. Chem. Biol.* **1998**, **2**:397-403.

In view of the above, the Applicants respectfully request that the Examiner withdraw the rejection for lack of enablement.

II. 35 U.S.C. § 112 SECOND PARAGRAPH REJECTIONS

The Examiner rejects claims 11 and 13 as being indefinite because:

> The expression "**M**" in claim 11 renders claims 11 and 13 indefinite. The expression "**M**" is not understood since "**M**" is not defined in the formula I. Therefore, the scope of the claims is indefinite as to the structural formula encompassed thereby. (Office Action p. 12)

The Examiner then responds to the Applicants' arguments saying:

> Applicant asserts that "The term "M" is defined functionally in claim 11 in that "wherein in vivo or in vitro compounds of formulae I and IA are converted to M-PO_3^{2-}, which inhibits FBPase". Contrary to Applicant's assertion, one of ordinary skill in the art could not ascertain and interpret the **metes and bounds** of the patent protection desired as to what "M" encompassed thereby, which may reasonably encompass widely varying groups.
>
> Given the fact that any significant structural variation to a compound would be reasonably expected to alter its properties, e.g., physical, chemical, physiological effects and functions. Therefore, the scope of clims is indefinite as to the composition encompassed thereby. (Office Action p. 12)

The Applicants respectfully traverse this rejection.

The term "M" is defined functionally in claim 11 in that "wherein *in vivo* or *in vitro* compounds of formulae I and IA are converted to M-PO_3^{2-}, which inhibits FBPase." In other words, M must be a group that *in vivo* exists as M-PO_3^{2-} and is an inhibitor of FBPase. As discussed above in Section III, there is nothing inherently wrong with defining a term through its function.

Additionally, the specification at p. 25, lines 21-28 explains what is meant by an FBPase inhibitor.

> Combinations of the invention include at least one FBPase inhibitor. In most embodiments, the combination will include one FBPase inhibitor.

> FBPase inhibitors used in the invention are compounds that can inhibit human FBPase activity (Examples A-B), inhibit glucose production from hepatocytes (Examples C-D), lower glucose levels in fasted animals (Examples E-G), or decrease blood glucose levels in diabetic animal models (Examples V and W). Preferred FBPase inhibitors are compounds that inhibit enzyme activity as determined by conducting *in vitro* inhibition studies (Examples A and B).

Like the applicant *In re Fuetterer*, the Applicants are not claiming insulin secretagogues and FBPase inhibitors, but instead a composition containing insulin secretagogues and FBPase inhibitors. Consequently, the Applicants need not list the name or structure of every compound capable of acting as an insulin secretagogue or an FBPase inhibitor.

Furthermore, the Applicants believe that a person of ordinary skill in the art could determine which compounds to use through routine experimentation, such as described in Examples A and B. With the use of high-throughput screening, there is nothing undue about the amount of experimentation needed to determine what compounds are encompassed by the claims. A person of ordinary skill in the art could ascertain the metes and bounds of the composition claimed.

In view of the above, the Applicants respectfully request that the Examiner withdraw the indefiniteness rejection.

III. 35 U.S.C. § 103 REJECTIONS

The Examiner has rejected claims 1-5, 11-18, and 20-45 under 103(a) as being unpatentable over Kasibhatla *et al.* and Melchior *et al.* The Examiner states:

> Kasibhatla et al. (WO 98/39342, WO 98/39343, and WO 98/39344) discloses that the instant particular compounds for example having the formula 1 in WO 98/39342, the formula 1 in WO 98/39343, the formula 1 in WO 98/39344, being FBPase inhibitors at the AMP site, are useful in a composition and a method of treating diabetes in a mammal. See WO 98/39342: abstract, page 1 lines 5-10, page 5-15-47 and claims 1-53; WO 98/39344: abstract, page 1, lines 5-10, page 6-36 and all claims therein; WO 98/39343: abstract, page 1 lines 5-10, page 6-75, and all claims therein.
>
> Melchior et al. teaches that the particular insulin secretagogue, sulfonylureas such as glyburide, is well known to be useful in a composition and in the treatment of diabetes in a mammal: See the abstract in particular.

The prior art does not expressly disclose that the employment of the particular FBPase inhibitor of Kasibhatla et al. in combination with that particular insulin secretagogue, sulfonylureas such as glyburide in a composition for the treatment of diabetes.

It would have been obvious to a person of ordinary skill in the art at the time the invention was made to employ the particular Fbpase inhibitor of Kasibhatla et al. in combination with particular insulin secretagogue, sufonylureas such as glyburide in a composition for the treatment of diabetes.

One having ordinary skill in the art at the time the invention was made would have been motivated to employ the particular Fbpase inhibitor of Kasibhatla et al. in combination with particular insulin secretagogue, sufonylureassuch as glyburide in a composition for the treatment of diabetes since both the particular Fbpase inhibitor of Kasibhatla et al., and in particular sufonylureassuch as glyburide are known to be useful in a composition and a method of treating diabetes in a mammal based on the prior art.

Therefore, one of ordinary skill in the art would have reasonably expected that combining the particular Fbpase inhibitor of Kasibhatla et al. in combination with particular insulin secretagogue, sufonylureas such as glyburide both know useful for the same purpose, i.e., treating diabetes, would improve the therapeutic effects for treating the same diseases, and/or would produce additive therapeutic effects in treating the same.

It has been held that it is prima facie obvious to combine two compositions each of which is taught by the prior art to be useful for same purpose in order to form third composition that is to be used for very same purpose; idea of combining them flows logically from their having been individually taught in prior art. See *In re Kerkhoven*, 205 USPQ 1069, CCPA 1980.

Thus the claimed invention as a whole is clearly prima facie obvious over the combined teachings of the prior art. (Office Action pp. 13-15)

The Examiner goes on to address the Applicants' arguments saying:

Applicant argues that "The cited art does not suggest that a combination of insulin secretagogues and FBPase inhibitors will result in any additive effect. A person of ordinary skill in the art would not randomly combine agents that show improved glycemic control and expect that such a combination would be successful".

> Applicant's argument has been considered but is not found persuasive. It has been held that it is prima facie obvious to combine two compositions each of which is taught by the prior art to be useful for same purpose in order to form a third composition that is to be used for the very same purpose; idea of combining them flows logically from their having been individually taught in the prior art.
>
> In the instant case, as discussed in the set forth 103(a) rejection above, both the particular FBpase inhibitor of Kasibhatla et al., and particular insulin secretagogue, sulfonylureas such as glyburide are known to be useful in a composition and a method of treating diabetes in a mammal based on the prior art.
>
> Therefore, one of ordinary skill in the art would have reasonably expected that combining the particular FBpase inhibitor of Kasibhatla et al. in combination with particular insulin secretagogue, sulfonylureas such as glyburide both known useful for the same purpose, i.e., treating diabetes, would improve the therapeutic effects for treating the same diseases, and/or would produce additive therapeutic effects in treating the same, absent evidence to the contrary.
>
> Therefore, motivation to combine the teachings of the prior art cited herein to make the present invention is seen. The claimed invention is clearly obvious in view of the prior art.
>
> The record contains no clear and convincing evidence of nonobviousness or unexpected results for the combination herein over the prior art. In this regard, it is noted that the specification provides no side-by-side comparison with the closest prior art. (Office Action pp. 15-16)(internal citations omitted)

The Applicants respectfully traverse this rejection.

The current invention is directed toward compositions containing insulin secretagogues and FBPase inhibitors. As the Examiner admits, "the prior art does not expressly disclose that the employment of the particular FBPase inhibitor of Kasibhatla et al. in combination with that particular insulin secretagogue, sulfonylureas such as glyburide in a composition for the treatment of diabetes." (Office Action p. 11)

Although improved glycemic control is a reported consequence of treatment with either insulin secretagogues or FBPase inhibitors, the combination of these two drugs is novel. The cited art does not

suggest that a combination of insulin secretagogues and FBPase inhibitors will result in any additive effect. A person of ordinary skill in the art would not randomly combine agents that show improved glycemic control and expect that such a combination would be successful.

The following quotation from Example X indicates that improved glycemic control that resulted from combining FBPase inhibitors and insulin secretagogues:

> This study indicates that combination treatment with an insulin secretagogue and an FBPase inhibitor significantly improved glycemic control over treatment with either agent alone. (p. 316, lines 32-34)

The following quotation from the specification also describes the improvements associated with the combination therapy:

> Based on the pharmacological profile of insulin secretagogues and FBPase inhibitors described above, a therapy in which insulin secretagogues are combined with FBPase inhibitors is effective across a broad patient population. In early stage diabetics, FBPase inhibitors and insulin secretagogues are both fully effective. Despite the well-characterized effect of insulin on hepatic glucose output, combination treatment of an insulin secretagogue and an FBPase inhibitor not only provided improved glycemic control in early stage diabetes (Example X), but also reduced the incidence of secondary failure commonly observed with insulin secretagogue monotherapy (Example Y). In advanced diabetics, insulin secretagogues have a high primary failure rate and are only partially effective, whereas the FBPase inhibitors maintain robust efficacy. The benefit of the combination in advanced diabetics is a significant decrease in the number of nonresponders to therapy and an overall increased degree of glycemic control. While the initial response of combination therapy in advanced diabetics may in large part be due to treatment with the FBPase inhibitor, blood glucose lowering improves pancreatic function and allows the insulin secretagogue to become more fully effective over time and in the long term thus provides improved response to the insulin secretagogue and enhanced glycemic control. (p. 207, line 18 – p. 208, line 3)

The glycemic control that resulted from the combination of this invention was significantly greater than that obtained by administration of either agent alone. (*see* Figure 1 p. 317) Although, a person of ordinary skill in the art might have expected that the combination of insulin secretagogues or FBPase inhibitors would result in glycemic control, they would not have known that the combination of this invention would result in significantly greater glycemic control than that obtained by administration

of either agent alone. Therefore, the improved significantly improved glycemic control obtained with the combination of insulin secretagogues or FBPase inhibitors was surprising and unexpected.

The Examiner states that the Applicants do not present a comparison with the prior art. In Example X, the Applicants do present a comparison of the combination versus the results with either compound alone.

The Examiner also contends that the Applicants do not present clear and convincing evidence of nonobviousness. The Applicants note that there is no clear and convincing standard.

Additionally, the combination of insulin secretagogues or FBPase inhibitors of this invention resulted in other surprising and unexpected benefits.

> Another important benefit of insulin secretagogue-FBPase inhibitor combination treatment is an unexpected beneficial effect on carbohydrate, and/or lipid, and/or protein metabolism.
>
> Another benefit of the combination therapy is that FBPase inhibitors can attenuate the side effects associated with insulin secretagogue therapy, and vice versa. A key consequence of insulin secretagogue therapy is hyperinsulinemia which results in the undesirable side effects of promoting weight gain, of exacerbating insulin resistance, and of predisposing patients to hypoglycemic episodes. Hyperinsulinemia may also be associated with increased risk of macrovascular disease. Insulin secretagogues can also overstimulate the pancreas and consequently promote beta cell degeneration and thus secondary failure. Likewise, FBPase inhibitors may have undesirable side effects in man. FBPase inhibitors may, for instance, cause a transient rise in blood lactate levels. As described in Example X, combination therapy of an FBPase inhibitor and an insulin secretagogue (glyburide) resulted in an unexpected attenuation of the blood lactate elevation caused by FBPase inhibitor monotherapy. (p. 208, lines 4-18)

In view of the above, the Applicants respectfully request that the Examiner withdraw the obviousness rejection.

IV. DOUBLE PATENTING REJECTIONS

The Examiner provisionally rejects claims 1-5, 11-18, and 20-45 under the judicially created doctrine of obviousness-type double patenting as being unpatentable over all the claims of copending Application No. 09/470649. The Examiner says:

> The claim of the instant application is drawn to employ the same Fbpase inhibitor in combination with insulin secretagogue, such as sulfonylureas, e.g., gylburide in a composition for the treatment of diabetes. Thus, the two compositions in the copending Application and the instant Application are seen to substantially overlap. (Office Action p. 17)

The Examiner then addresses the Applicants' previous arguments saying:

> Applicants argue that the agents claimed in the copending Application and the present Application for in treating diabetes operate by different mechanisms. However, the particular insulin secretagogue, sulfonylureas, e.g., glyburide, would be reasonably interpreted as an INSULIN SENSITIZER FOR THE TREATMENT OF DIABETES, as claimed in the copending Application. Thus, the two compositions in the copending Application and the instant Application are seen to be obvious to each other. (Office Action p. 18)

The Applicants respectfully traverse this rejection.

The Applicants again note that Application No. 09/470649 has been allowed and is now U.S. Patent No. 6,756,360. Since the Examiner has not indicated that she has reviewed the issued claims and an obviousness-type double patenting rejection is based on a comparison of the claims of the issued patent to the pending claims, the Applicants respectfully request removal of the provisional rejection and ask the Examiner to reevaluate her rejection based on the issued claims.

The Applicants do wish to point out that a person of ordinary skill in the art would not reasonably expect that the successful combination of one agent useful in the treatment of diabetes with an FBPase inhibitor would mean that combining another agent useful in treating diabetes with an FBPase inhibitor would be successful, particularly when the agents useful in treating diabetes operate by different mechanisms.

Isulin secretagogues and insulin sensitizers operate by different mechanisms as noted in the specification of the current Application:

> The **insulin secretagogues** target defects in insulin secretion by the pancreas, defects which are typically observed in diabetics. (p. 2, lines 18-20)(emphasis added)
>
> **Insulin sensitizers** are another class of oral agents. Peroxisome proliferator-activated receptors (PPAR-gammas) appear to be the target of the most recently introduced class of antidiabetic agents, the insulin sensitizers. These drugs are reported to enhance insulin-mediated glucose

disposal and inhibition of hepatic glucose output without directly stimulating insulin secretion. (p. 2 line 30 – p. 3, line 3)(emphasis added)

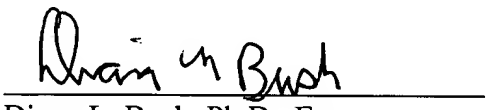
The Applicants also respectfully ask the Examiner to contact the undersigned if she wishes to maintain this rejection.

CONCLUSION

In view of the above remarks, it is believed that the application is in condition for allowance, and such action is respectfully requested at the Examiner's earliest convenience.

Respectfully Submitted,

Date: May 27, 2005

By: 

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New technologies for high-throughput screening

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To screen efficiently the millions of compounds that are synthesized using combinatorial and automated methods, dramatically improved assay technologies are currently needed. In 96-well microtiter plates, nonradioactive techniques (primarily fluorimetric) and cell-based functional methods have moved to the cutting edge, while clever assays that extract information from large bead-based combinatorial libraries have begun to show considerable promise. In the future, miniaturized assays that break out of the 96-well format will be enabled by innovative technologies for high-throughput screening.

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Abbreviations

CL	chemiluminescence
DTTA-PITC	N^1-(p-isothiocyanatobenzyl)-diethylenetriamine-N^1,N^2,N^3,N^3-tetraacetic acid
FP	fluorescence polarization
FRET	fluorescence resonance energy transfer
HTS	high-throughput screening
HTS-NT	high-throughput screening new technologies
Ln-TRF	time-resolved fluorescence of lanthanide ions

Introduction

High-throughput screening (HTS) is a well-established method for identifying useful, novel chemical structures. Because of new synthesis technologies such as combinatorial chemistry and automated synthesis, the numbers of new molecules available for screening have exploded in the past few years. Furthermore, a growing number of new targets have begun to emerge from genomics efforts. New technology development in HTS is propelled by the need to evaluate more compounds active against more targets.

Currently, HTS involves a relatively straightforward extension of laboratory-scale assays. Depending on the assay, the adaptation of an assay to HTS can involve either miniaturization (typically, to a 100 µl microtiter plate assay) or automation (generally, attempting to mimic as closely as possible the actions of a researcher in a laboratory assay), or both. Accordingly, the sampling rate that distinguishes 'high-throughput' from slower screening depends on the difficulty of the assay. A typical enzyme-based screen can generally achieve a throughput of 3000 to 5000 samples per day for HTS, while cell-based or other nonbiochemical screens may achieve only a few hundred samples per day to be classified as 'high-throughput'.

This review will cover HTS technologies that are currently in use, as well as those that are in the process of being implemented. Improvements in HTS fall into several categories: simplification of assay development; reduction in per-assay costs; and improvement in sample throughput. Consequently, we shall avoid the prefixes 'ultra-' and 'very' which specifically refer to improvement in sample throughput, and use the broader suffix '-NT' to denote the new technologies, in order to define the field as HTS-NT. We have divided the field into three areas of interest: HTS-NT for liquid-phase assays that broaden the applicability of 96-well microtiter plates; HTS-NT for solid-phase binding assays that exploit the solid-phase syntheses characteristic of some combinatorial libraries to improve throughput; and miniaturization technologies for HTS-NT that enable liquid-phase assays in volumes of one microliter or less.

The 96-well microtiter plate technology

Nonradioactive methods

Many laboratories have begun to favor assays that avoid the use of radioactive isotopes. This aversion is due not only to the cost of reagents and to the cost per assay, but also to the inherent limitations on miniaturization of radioactive assays: An assay with, say, a 1000 cpm signal in 100 µl would necessarily have only a 10 cpm signal in a 1 µl assay, requiring 10000 times as long to count to the same level of accuracy. The principal alternatives to radioactivity are fluorescence and chemiluminescence.

Over the years, several fluorescence methods have been developed to address a wide range of biological assays. In general, using simple fluorescence does not provide adequate performance for HTS, even though it is an inherently sensitive technique. In principle, a single fluorescent molecule can produce thousands of photons such that, in favorable cases, limits of detection have been extended to the single-molecule level. Its primary drawback, however, is a susceptibility to background effects, both from the biological milieu and from photophysical effects such as light scattering.

Table 1 compares nonradioactive detection methodologies that have been applied to HTS. One extremely versatile and sensitive method that serves broadly as a replacement for radioactivity is based on the time-resolved fluorescence (TRF) measurements of the rare earth lanthanide ions (LnTRF) such as europium (Eu). The first application of LnTRF to screening involved antibodies labelled with lanthanides for use in sensitive immunoassays (for

recent references see [1,2]). Because the Eu^{+3} label is at least as sensitive as ^{125}I, which is commonly used in radioactivity assays, this technique has found increasingly broad application as a replacement for radioactivity in HTS assays. Furthermore, many types of assays that have been developed using radioactive labels can be switched to lanthanide-based assays, simply by using different labelling reagents. For example, chemical labelling of free amines has traditionally been carried out using [^{125}I]Bolton–Hunter reagent [N-succinimidyl-3-(4-hydroxy-5-[^{125}I]iodophenyl)propionate] for which the amine-reactive europium chelate Eu^{+3}-DTTA-PITC serves as a straightforward replacement. In addition, a solution that dissociates europium from the complex in order to enhance the lanthanide fluorescence acts as a replacement for scintillation fluid. Examples of radioactive assays that have been successfully converted to lanthanide assays include those for several types of receptors (both direct ligand labelling [3•] and Eu^{+3}-labelled streptavidin-based detection of biotinylated targets [4,5] and tyrosine kinase assays (using Eu^{+3}-labelled antiphosphotyrosine antibodies [6]). The lanthanide-based system is, however, restricted to assays of pH >7, which ensures the integrity of the chelate.

Further development of LnTRF has been directed toward 'all-in-one' reagents that incorporate the properties of the enhancement solution into the the Eu^{+3} complex, in order to shorten the total time of the assay and to provide a more stable, nondissociable cryptate complex. Achieving comparable sensitivity with these cryptates has proven to be difficult, however, due to the interaction of a single europium ion with multiple excitation 'antennae' when dissociated [7]. Recently, a system has been developed that increases sensitivity by using a high-intensity laser to excite the cryptate [8]. Assays based on FRET (fluorescence resonance energy transfer) from a caged Eu^{+3} to allophycocyanin (APC) further expand the range of the LnTRF method. In this manifestation, it has found many applications in screening, provided a suitable site can be identified for incorporation of the second (APC) label. Incorporation of APC can either be achieved directly (e.g., through a reactive labelling reagent) or using labelled antibodies directed toward a non-obtrusive site in the target [9••,10].

FRET is increasing being used in nonradioactive screening methods [11,12]. A major consideration in choosing an assay based on energy transfer is the distance change that is induced upon ligand binding or enzyme turnover. For energy transfer to be possible, the distance must be less than about 40–50 Å [13]. To put this distance in perspective, 40 Å is approximately the diameter of a protein molecule with molecular weight 26 000 Da. In certain instances (e.g., small peptides that can act as protease substrates), this distance is achievable with mathematical certainty. FRET is of particular utility in assays of proteases, since doubly labelled peptide substrates are generally synthetically accessible and can be obtained from a number of contract suppliers. In other cases (e.g, the binding of a protein ligand to its receptor) the efficacy of the labelling geometry is far less certain.

The use of fluorescence polarization (FP) in HTS applications is growing as a result of the availability of reagents and the development of a 96-well plate reader system [14,15•,16•]. The technique is well founded in the diagnostics area, serving as the basis for many marketed diagnostics. In this technique, binding events are detected as a loss in rotational mobility of a fluorescence group. The advantages of FP are that only one fluorescent label is needed, and a homogeneous assay can be performed in the presence of background fluorescence. Since the polarization shift taking place upon excitation of the fluor

Table 1

Comparison of methodologies for nonradioactive detection.

Method	Advantages	Disadvantages
Straight fluorescence	Simple labelling Simple detection	Sensitivity limited by biological background
Lathanide time-resolved fluorescence (LnTRF)	Sensitive Simple labelling	pH > 7 needed to maintain chelate Requires enhancement solution
Fluorescence resonance energy transfer (FRET)	Large Stokes' shift Homogeneous	Two labelling steps needed Sensitive to distances on the molecular scale
Fluorescence polarization (FP)	Simple labelling Homogeneous	Sensitive to distances on the molecular scale
Homogeneous time-resolved fluorescence (HTRF)	Sensitive Homogeneous Large Stokes' shift	Two labelling steps needed Sensitive to distances on the molecular scale
Chemiluminescence (CL)	Very sensitive Few interferences	Specialized labelling chemistry needed Not versatile

is proportional to the fraction of the total fluor in the bound state order, assays may need to be performed in receptor excess, a situation that is not always practical. Enzymes (primarily proteases, but also kinases and phosphatases) can also be assayed using FP. Compared with FRET, FP is more useful when the targets are large, since larger changes in rotational mobility can generally be observed.

New and potentially interesting approaches for time-domain fluorescence measurements (or two-photon induced fluorescence) are being developed to improve sensitivity under a variety of experimental circumstances [17••,18]. These homogeneous time-resolved fluorescence (HTRF) approaches may be applicable to HTS-NT, but generally require specialized reagents for optimal application as well as new equipment that has yet to be developed. Additionally, Eigen and Rigler [19,20] have suggested that new methodologies employing advances in single-molecule detection can be applied to HTS-NT. It is not yet clear whether limitations in the speed of data collection (at low fluor concentrations) or the saturation of the detection stream (at high fluor concentrations) will limit the range of applicability of HTRF too severely.

Advancements in fluorescent reagents have also been helpful in HTS-NT. Labels that are red-shifted can be distinguished from the biological background (excitation >520 nm) more easily, and are consequently helpful where increased sensitivity is important. The cyanine-based dyes first synthesized by the Waggoner group at the University of Pittsburgh [21,22], are particularly useful in this regard. Reagents that fluoresce in the infrared are also being developed, but have not found significant application in HTS to date, primarily due to lack of available detectors, and insufficient experience with IR labels on the part of biological researchers [23].

Chemiluminescence (CL) is another photometric technique that is applicable to HTS. Detection of CL is a convenient adjunct to fluorescence, since most plate readers capable of measuring fluorescence will measure luminescence as well. This technique has been used predominantly with luciferase reporter genes in cell-based assays and in high-sensitivity enzyme-linked immunosorbent assays (ELISA) employing chemilumogenic substrates for alkaline phosphatase and horseradish peroxidase [24,25]. Recently, electrochemically generated chemiluminescence has been applied as a sensitive and versatile means of detection, using special, redox-active labels [26•,27,28]. To date, however, this technique has been applied primarily in immunoassay-based detection.

Cell-based functional methods

Functional methods for screening receptor-mediated phenomena have many advantages over traditional receptor binding assays. Essentially, functional screens enable the researcher to discriminate between different binding modes (agonist versus antagonist), as well as to broaden the target base to multiple components of a signalling cascade, regardless of the degree of biochemical characterization of the pathway. Cytofunctional assays, in particular, are finding increasing application to HTS-NT.

One promising methodology is a high-throughput screen based on calcium mobilization. This well-established cytometric assay was developed in 1989 in the Tsien laboratory at the University of California at San Diego [29], but has not been used effectively for HTS because the calcium response is short-lived (on the order of seconds). The development of a high-speed, high-sensitivity imaging system [30], now commercially available, has helped to overcome the limitations of calcium detection and made it a practical HTS methodology. This imaging system is able to collect parallel image data at speeds up to 10 frames per second, at sensitivities that allow detection of calcium transients in cell populations. Concurrent liquid handling and imaging was another key development for the success of this technique.

Another approach toward measurement of cellular assays, particularly assays of membrane potential and intracellular calcium release, is based on the use of a high-intensity laser source with narrow depth-of-field optics which helps to eliminate background. This instrument, known as FLIPR (for fluorescence imaging plate reader), permits rapid parallel stimulation and imaging with high sensitivity [31••]. Like the method described above, this combination of fluidics and imaging allows for high throughput despite the transience of the response.

The yeast *Saccharomyces cerevisiae* has also been useful in developing new HTS methods. It is relatively easy to transfect yeast with human DNA encoding receptors or other components of signal transduction pathways and this fact has been used to develop an HTS screening system [32]. The signalling pathways in yeast and humans are sufficiently related to permit functional evaluation of human receptors in yeast cells. This method has found particular use in the analysis of human G-protein-coupled seven-pass transmembrane receptors which work well in yeast pathways and provide convenient readouts such as growth (inhibition or promotion) or transcription of reporter genes. Because of the straightforward manipulation of genetic information in yeast, it is relatively simple to generate a family of strains that differ by a single human gene, to facilitate the analysis of screening data. These HTS assays have identified molecules that interact with the human receptors, as well as peptide ligands for orphan seven transmembrane receptors (receptors for which ligands are as yet unknown) [33•].

A technique that involves ligand-dependent transformation of mammalian cells has also been developed. In this method, stimulation of any one of a number of receptors that are transiently expressed in NIH 3T3 (fibroblast) cells confers a growth phenotype [34,35].

Compounds that interact with the stimulated receptor can be identified colorimetrically, because the reporter gene encoding β-galactosidase is coamplified during cell proliferation. This method is particularly useful in the functional classification of compound libraries containing both agonists and antagonists.

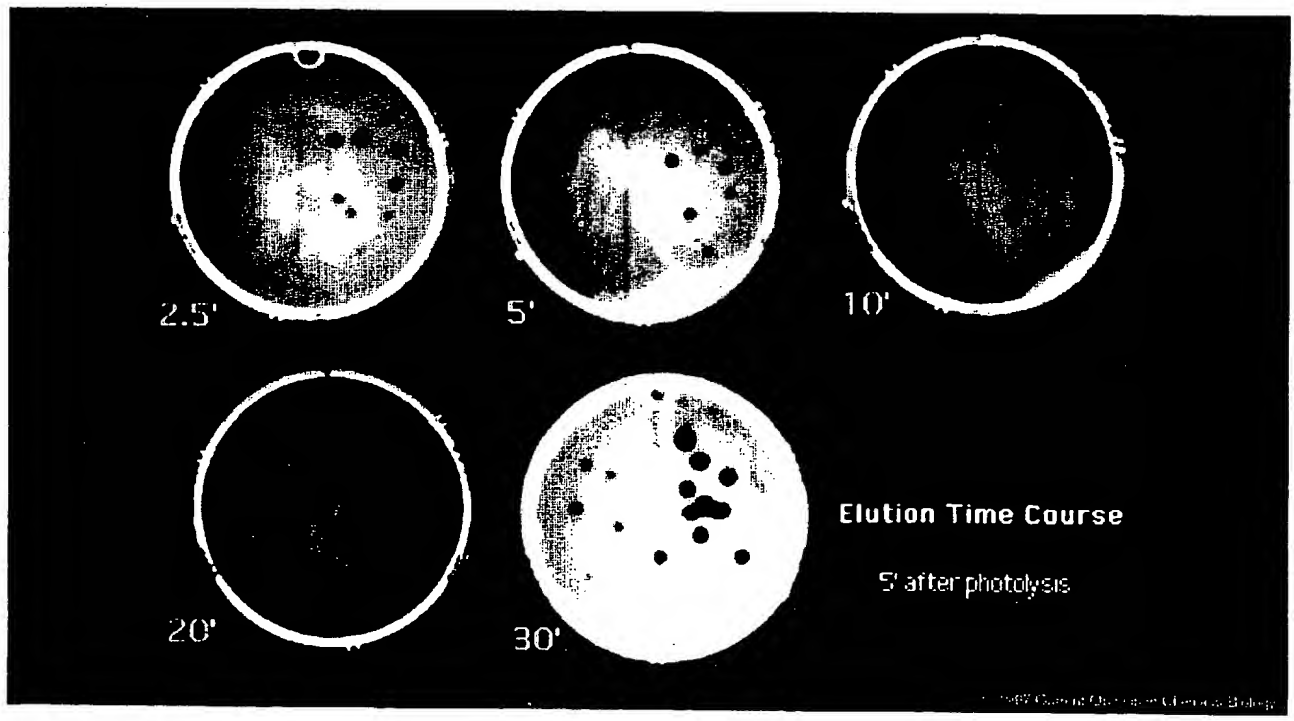
Methods for screening combinatorial libraries

The advent of solid-phase synthesis as the preferred methodology for generating large combinatorial libraries has presented an opportunity to exploit the immobilization of small molecules to facilitate assay development. Among the early examples of the effectiveness of large libraries as discovery tools was the demonstration that antibody epitopes can be defined by screening combinatorial libraries of peptides. This methodology has led rather naturally to recent developments that involve solid-phase synthetic libraries and their interactions with protein domains [36,37••].

Since solid-phase binding assays do not necessarily reflect the properties of small molecules in solution, additional methodologies have been developed that involve release of the small molecules at precisely defined times and locations. One example of this type of assay is the so-called 'field format' enzyme assay (Fig. 1), in which beads containing a solid-phase combinatorial library attached with a photocleavable linker are immobilized in agarose containing the enzyme of interest.

Additional exploitation of the solid phase has involved selection of compounds through their ability to bind to an immobilized target. Depending on the types of compounds and binding conditions used, this technique can permit the selection of the highest affinity member of a library (since, under excess ligand conditions, this member will displace weaker binding molecules). Alternatively, fractionation and/or direct characterization of the bound molecules can lead to a whole family of binding molecules to allow for the generation of a structure-activity relationship (SAR). This technique has found most utility in identifying peptides that bind specifically to other proteins, for example, MHC class II molecules ([38] and references therein) and phosphotyrosine-binding domains [39].

Figure 1



Field format assay of carbonic anhydrase (M Traversari, T Nichols, CD Carroll, J Burbaum, D Chelsky, unpublished data). The assay was set up in two steps. First, a layer containing bovine carbonic anhydrase (0.1 μM) and low-melt agarose (SeaPlaque™, 0.8%) in sodium phosphate buffer (50 mM, pH 7.4) was layered over two types of beads containing covalently attached carbonic anhydrase inhibitors of the aryl sulfonamide class. On the left side as shown, beads containing an inhibitor with a K_i in solution of 660 nM were used, while on the right side, beads containing an inhibitor with a K_i in solution of 4 nM were used. After the first layer solidified, a second layer containing the fluorogenic substrate fluorescein diacetate was added. A series of photographs taken after the second addition, showing the development of zones of inhibition as a function of inhibitor potency is shown. The numbers at the lower left of each field indicate time in minutes after photolysis of the inhibitors.

Fluorescence-activated bead sorting holds great promise in identifying individual beads that contain compounds of interest. This methodology has been limited to date by technical issues. Commercial cell sorters need to be re-engineered to accommodate bead sorting. The full potential of this strategy may not be realized before the development of specialized instruments designed around the flow characteristics of combinatorial library beads.

Miniaturization

Needs

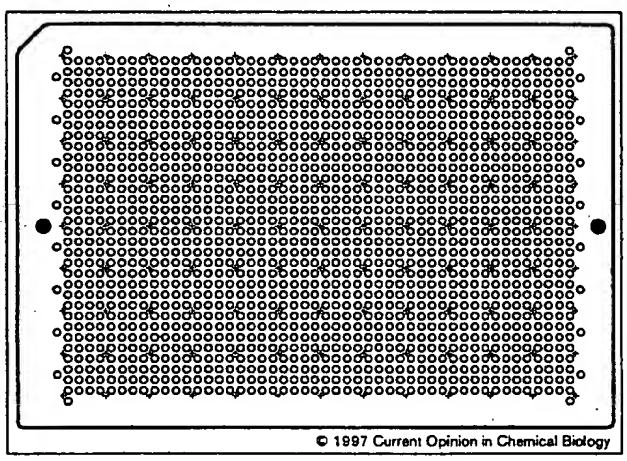
The technological advances of HTS-NT have resulted in assays that are quicker to set up and execute and are beginning to provide the means to release the full potential of combinatorial chemistry and functional genomics for drug discovery. Miniaturization is vital for full actualization because, in simple terms, the total number of assays required is the product of the number of targets and the number of compounds available for testing. For example, suppose that a full screening effort for a major pharmaceutical company in the year 2000 would require the survey of a deck of 10^6 compounds against 200 targets per year. This supposition approximates a significant increase in both the compound deck and the target pool. In this scenario, 2×10^8 assays of 100 µL, each containing 10 µM test compound with nominal molecular weight of 500 g/mol, would require roughly two million microtiter plates, 20 000 liters of each target solution, and 100 grams of each compound per year. Clearly, the expenses required to extract all the information needed will be exorbitant without a technological overhaul of the screening process.

Due to the diverse biochemistry of potential targets, which range from high turnover enzymes to low copy number receptors, not all assays will be amenable to miniaturization. Nonetheless, miniaturization (to the extent that assays remain competent and straightforward to establish) will be embraced in the future. There are presently two broad efforts in miniaturization, classified by the type of container that is needed. In the simpler case, the sample container is an open vessel similar to a microtiter plate well, only smaller. Beyond a certain point, however, miniaturization in an open vessel is impractical because of rapid evaporation of the sample. Thus, miniaturization below the microliter scale requires a closed vessel, which raises issues of loading samples and biological reagents into a tiny chamber.

Open vessel

For conventional well shapes (round- and flat-bottomed), the lower limit for an open container in a laboratory environment is on the order of one microliter (J Burbaum, R Affleck, unpublished data). In this format, more rapid analytical sampling is achieved by packing the microliter-volume wells more densely, in other words, by having more wells per unit area of the plate surface. To maintain compatibility with the 96-well world, the plate density should follow the geometric series $N = n^2 \cdot 96$ where N is the number of wells on the plate and n is an integer, which describes the possible packing densities in a rectilinear array. The balance point of these two limitations, microliter volume and suitable packing density, suggests a plate with 1536 wells (n = 4), 1–2 µl in volume. A commercial plate having these properties is the 1536-well HTS plate shown in Figure 2.

Figure 2



1536-well plate. Each well in a standard 8 row × 12 column 96-well plate has been replaced by a grid of 16 shallow wells of approximately 2 µl total volume. In addition, eight control wells on 9 mm spacing have been situated at the ends of the rows, and four standard wells have been placed at the top and bottom of the first and last columns. Through-holes (●) that permit alignment of the plate in a plate holder have also been included.

New techniques for detection of fluorescence in the 1536-well format are needed. Conventional plate readers for fluorescence typically read one well at a time, but each 1536-well plate would take about sixteen times longer to read. Because of increased throughput requirements, serial sampling will be unacceptably slow unless substantially accelerated. A more promising approach involves the use of fluorescence imaging, using (for example) sensitive camera-based technologies, to collect data from all the wells at once. Fluorescence imaging is a technique that has seen recent specialized applications in HTS (see above). More general fluorescence imaging techniques being developed will require the development of new strategies to allow for the effects of miniaturization. In particular, the development of robust labelling reagents that fluoresce at longer wavelengths will be needed, with enough versatility to allow for many kinds of fluorescence spectroscopy.

The use of microscopy as a detection tool is another technique that offers more sensitivity. Fluorescence microscopy is a well-established analytical method in cell biology and pathology that is also quantifiable. Until recently, however, limitations in data processing speed

have limited its usefulness in HTS. Clearly, assays that require increased sensitivity would be amenable to microscopy techniques, since microscopy has traditionally yielded fluorescence measurements of single cells.

Finally, techniques for using cell-based assays are needed. HTS will benefit from the miniaturization of the calcium flux and ion channel instrumentation described earlier; in order to exploit the full spectrum of targets, however, the development of new reporter genes, both for new enzymes and for new fluorescent proteins, will be needed. The utility of various reporter genes in HTS-NT was reviewed recently [33], so will not be covered further.

Closed vessel

In order to develop assays with volumes significantly less than a microliter, a dramatic rethinking of conventional assay strategies is required. This conceptual shift is caused by the necessity of enclosing the assay sample completely to prevent evaporation. Volumetric delivery of fluids in nanoliter volumes without cross-contamination is clearly a problem that must be resolved before HTS-NT in enclosed containers is considered practical. Two new companies have accepted this challenge, and have taken promising approaches toward defining HTS in nanoliter volumes: Caliper Technologies (Pao Alto, California), and Orchid Biocomputer (Princeton, New Jersey).

Caliper's technology uses advanced capillary microfluidics driven electrophoretically to enable rapid and reproducible transport of fluids capable of carrying an electrical current. Recent advances in manufacturing technologies have facilitated the development of complex microfluidic circuits (for a recent review see [40•]) that have been described as laboratories on a chip. To be applicable for HTS-NT, however, assays need to be reworked using electrophoretic or chromatographic separations. Much work is needed to develop robust and versatile separations for biological assay samples in these small volumes, but the payoff in miniaturization and assay acceleration would be striking, particularly with assays involving kinases and proteases for which separations-based assays are currently used.

Orchid's technology integrates synthesis and analysis of library compounds in nanoliter volumes. The challenge of aliquoting organic solvents for synthesis on a nanoliter scale has been met using a proprietary electronic pump that can be used with any solvent. Using fabrication techniques pioneered in the semiconductor industry, nanoliter reaction and analysis vessels are being integrated, such that a single four-inch silicon wafer will support 10^5 separate syntheses/bioassays. Because separations are not required for a successful assay, this technology will provide a strong complement to the miniaturized separations afforded by Caliper's 'lab-on-a-chip' approach. This technology, when mature, will allow for the simultaneous capture of data using a CCD charge-coupled device chip of identical dimensions, allowing for the analysis of many kinds of HTS assays in a rapid, highly-parallel fashion.

Conclusions

Over the next few years we should see a dramatic increase in the ability to analyze large sets of compounds against a large number of targets, as the new technologies for HTS described in this review come into routine use and others not anticipated here are invented. The reason behind this intense effort is obvious: the payoff in terms of new pharmaceuticals discovered will be well worth the investment in new technologies, and will provide the next step forward in the biotechnology revolution. The impact of facilitated compound characterization afforded by HTS-NT will also be significant, with new and more precise tools for dissection of cellular processes becoming readily accessible. New fluorescent reagents, more versatile labelling kits, and miniaturization of assays will all play a role in driving the technology. We can not yet predict the breadth of the impact, but can only predict that the changes in compound discovery methodologies will be fundamental.

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Fluorescence correlation spectroscopy: lead discovery by miniaturized HTS

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Miniaturized high-throughput screening offers great promise for increasing the productivity of the pharmaceutical drug discovery process. By monitoring interactions of single molecules in femtoliter volumes, fluorescence correlation spectroscopy (FCS) offers the highest potential as the detection technique in the nanoscale. The authors (Box 1) summarize the current status of practical experiences with FCS assays for HTS and explore the scope for further developments.

Box 1. Contributing research teams

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Three new scientific disciplines show the highest promise of fulfilling the need for increasing the predictability and for lowering the overall attrition rate of the drug discovery process.

- Functional genomics was invented to generate new innovative molecular targets
- Combinatorial chemistry provides increasingly efficient ways to generate molecular diversity with which to probe the targets
- High-throughput screening (HTS) platforms provide efficiency and quality in finding potential lead compounds

HTS within most pharmaceutical companies currently involves performing several million assays per year. Meanwhile, HTS has become a discrete discipline assimilating biochemistry, biophysics and cell/molecular biology combined with detection/liquid-handling technologies and automation processes. Testing a vast number of synthetic compounds and natural products against a target of therapeutic interest has imposed an ever increasing demand for short assay turnaround times, fast and high quality lead validation, immediate profiling of potential lead compounds, use of very diverse sample collections (e.g. discrete compounds, microbial extracts and combinatorial libraries), and saving of compounds, biological tools, reagents and

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waste (especially radioactive). In order to fulfil these requirements a screening concept based on homogeneous, automated assays in a miniaturized format that provides the highest possible mechanistic information (kinetic and thermodynamic) seems most appropriate.

Fluorescence spectroscopy

The only detection technique available that combines homogeneous mixtures of reagents, high sensitivity, true equilibrium in complexation reactions and a wide range of solution conditions is fluorescence spectroscopy. The picosecond to microsecond timescale of the emission of photons corresponds to the timescale of many dynamic events, including macromolecular rotational diffusion, solvent reorientation, energy transfer or motion of domains. By choosing the appropriate fluorescence label and strategy, fluorescence can provide information on size, distances, ligation state, conformational rearrangements and sample heterogeneity. Many pharmaceutical companies are increasingly developing and operating fluorescence-based HTS screens, typically using time-resolved or continuous wave fluorescence resonance energy transfer, fluorigenic enzyme substrates or fluorescence anisotropy, prior to and in preparation for the ultra-HTS screening era.

All macroscopic fluorescence methods, whether based on intensity, anisotropy or lifetime detection, average the emission signal from all excited molecules in the cuvette or well (ensemble averaging techniques). With these techniques the fluorescent signal typically decreases and the assay variability (coefficient of variation) increases as assays are conducted in 1–10 μl assay volumes (c.f. 50–200 μl volumes for conventional microtiter plate assays). Therefore, the current limit for the miniaturization of many types of assays is ~10–20 μl using commercially available readers. The only technical possibility to overcome the finite resolution dictated by the level of background noise in average intensity measurements is to record signals from single molecules. Such a measurement is feasible with a detection volume that is small enough to host, on average, only one or a few particles. The time-dependent fluctuations of (fluorescent) particles with different molecular properties, like molecular weight, translational and rotational diffusion time, colour and fluorescence lifetime, potentially provide all the kinetic and thermodynamic information required to study complex molecular interactions.

Fluorescence correlation spectroscopy

With the technical possibility to focus a laser beam to femtoliter volumes, such a new fluorescence technique, fluorescence correlation spectroscopy (FCS), finally became a practical possibility after it had been established in principle about 20 years ago[1,2]. Although the fundamental principles behind FCS are described elsewhere[3–9], a very brief summary of the basic technology is included here.

Conventional FCS takes advantage of differences in the translational diffusion of large versus small molecules. Each molecule that diffuses through the illuminated confocal focus gives rise to bursts of fluorescent light quanta during the entire course of its journey, with each individual burst being registered. The length of each photon burst corresponds to the time the molecule spends in the confocal focus. The photons emitted in each burst are recorded in a time-resolved manner by a highly sensitive single-photon detection device. This detection method achieves single-molecule sensitivity, but the fact that diffusion is a random process requires that the diffusion events for a minimum ensemble of molecules must be averaged to achieve statistically reliable information. The detection of diffusion events enables a diffusion coefficient to be determined. This diffusion coefficient serves as a parameter to distinguish between different fluorescent species in solution; for example, between free or bound ligand. In screening, the diffusion coefficient can be used to determine such factors as concentration or degree of binding. In addition, confocal optics eliminate any interference from background signals and allow homogeneous assays to be performed. FCS measurements are conducted in seconds making the technology ideally suited for high-throughput applications.

We emphasize five points relevant to miniaturized HTS:

- FCS is effectively insensitive to miniaturization because of the small size of the confocal detection volume. With less than a femtoliter (10^{-15} liter) as detection volume FCS is the ideal method for nanoscreening.
- By detecting the temporal fluorescence intensity fluctuations caused by the diffusion of single molecules in and out of a laser focus, FCS measurements contain all the spectroscopic information required to monitor changes in the molecular state and number of fluorescent molecules. In addition to the increase in translational diffusion time of a molecule by binding to a substrate or the decrease of translational diffusion time by cleavage reactions interpreted from the FCS signal, detection techniques

Box 2. The EVOscreen™ platform

The FCS and FCS-based assays described above were developed on an extended, FCS⁺plus capable reader based on the Zeiss-Evotec Confocor™ and run on separate pipetting, dispensing, storage and FCS detection and data evaluation software modules. In the meantime all individual components have been combined to form the EVOscreen™ system, a unique platform integrating highly sensitive detection technology and high-precision liquid handling systems in a modular system which will be capable of 100,000 assays per day. The core elements of the modular system are:

- A high performance confocal fluorescence detection unit usable in either a single channel or multi-channel mode
- Proprietary signal processing protocols based on FCS and related single molecule-based confocal fluorescence methodologies
- A miniaturized, automated liquid handling system for nano- to low-microliter volumes (including pipetting, dispensing and compound retrieval)
- A rapid, miniaturized multi-replica compound repository providing the link between traditional single compound library formats and EVOscreen™
- A micro-separation device (HPLC) coupled to the detection system
- A scanner and picker device for the analysis of combinatorial libraries and for functional genome analysis

monitoring intensity, particle number, anisotropy, cross correlation (energy transfer) and lifetime are technically feasible. At a mature state of development, FCS may therefore be expected to cover all traditional fluorescence techniques in one measurement with single-molecule resolution. In HTS the high information content also improves well-to-well and assay-to-assay quality control.

- FCS, as a diffusion method, is the only technique currently known that determines the concentration of interaction partners free of artefacts.
- The combination of 'true' concentration measurements with the detection of up to five different molecular parameters from one experiment allows molecular interactions to be mechanistically characterized during or immediately after the HTS run (see CD45 example below).
- Improvements in FCS optics have reduced read times to one or a few seconds per well for many types of assay, such that FCS can deliver the throughputs necessary for HTS screening (>20,000 assays day^{-1}).

Evotec Biosystems in Hamburg was founded in 1993 to exploit the possibilities of FCS in biology and medicine. Since 1996, in a milestone-driven collaboration, at first with Novartis Pharma and, since 1997, also with SmithKline Beecham Pharmaceuticals, the activities at Evotec have been concentrated on lead discovery. The EVOscreen™ platform – a modular, miniaturized ultra-HTS system based on FCS and Evotec's proprietary FCS-related single-molecule detection technology (FCS⁺plus) – is operational as of April 1998. In this review, Novartis, SmithKline Beecham and Evotec jointly report on a selected series of FCS-based assays that were developed to run on the EVOscreen™ system (Box 2).

Examples of FCS-based assays

Between the three companies, over 50 FCS-based assays including examples from many classes of target proteins typically encountered in the pharmaceutical industry were developed during the past two years. Outlines of some of these are given below. In addition, for one example (CD45 phosphatase), we describe in detail the steps from assay development through to the operation of a miniaturized prototype HTS screen.

Topoisomerase–DNA binding

Topoisomerases catalyse changes in the topology of supercoiled DNA during its replication, transcription and recombination. Inhibitors of human topoisomerases have been shown to be useful anticancer agents. Here, we developed an FCS assay to screen for inhibitors of DNA binding to *E. coli* topoisomerase I (Topo-I) and thus identify potential novel antibacterial agents. Topo-I is capable of binding relatively short fluorescent, single-stranded DNA oligonucleotides (e.g. TMR-22mer, M_r = 8 kDa). This results in a 13-fold increase in the molecular mass of the DNA–Topo-I binary complex (Topo-I, M_r = 97 kDa) relative to free TMR-22mer, and a corresponding increase in diffusion time measured by FCS (t_{DNA} = 237 μs, $t_{DNA-Topo-I}$ = 480 μs; Fig. 1a). The K_d for DNA binding determined by equilibrium titration (25 nM, Fig. 1b) is consistent with that determined by fluorescence anisotropy (35 nM; data not shown). Furthermore, the K_d determined for fluorescein-labelled DNA (13 nM) using assay volumes of 20 μl and 1 μl are identical (Fig. 1c) and consistent with that determined by non-FCS techniques (10–25 nM). These data exemplify the inherent insensitivity of FCS to miniaturization in the 1 μl range.

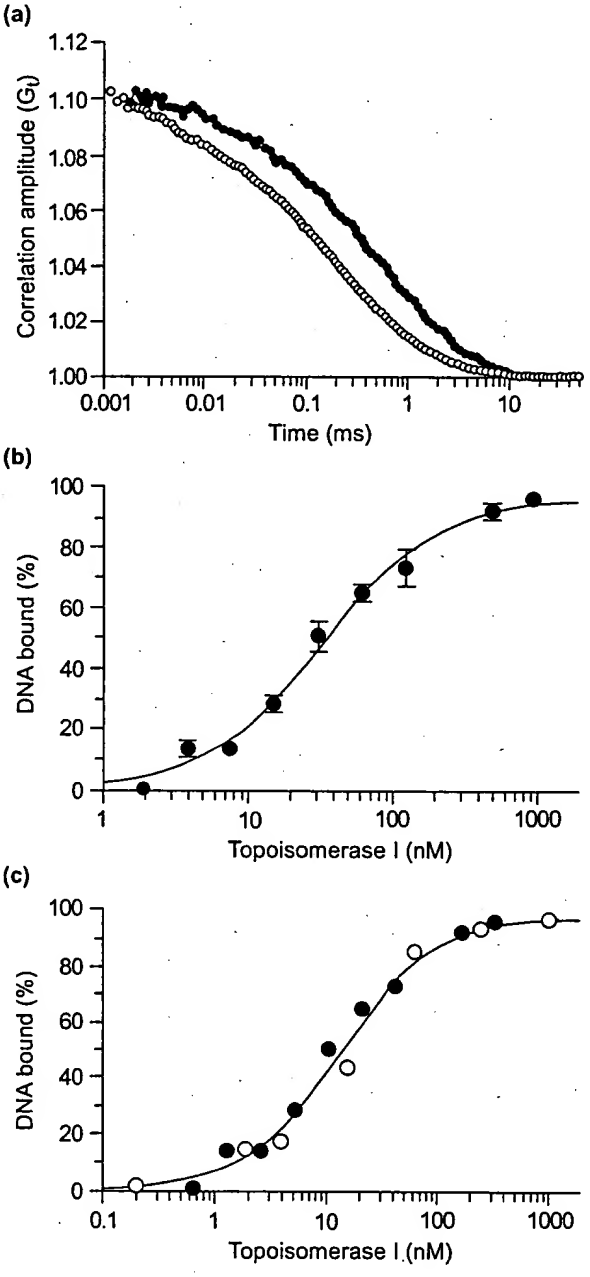

Figure 1. *FCS assay for DNA binding to* Escherichia coli *topoisomerase I (Topo-I). (a) Correlation curves of free (white circles) and bound (black circles) TMR–DNA. (b) TMR–DNA (10 nM) binding monitored by FCS in 20 μl assay volume (K_d = 25 ± 5 nM). (c) Fluorescein-DNA binding to Topo-I monitored by FCS in assay volumes of 1 μl (white circles) and 20 μl (black circles) (K_d = 13 ± 3 nM). Error bars show SD.*

Src-SH2–phosphotyrosine-peptide binding

Src is a membrane-associated protein tyrosine kinase containing an SH2 domain which recognizes and binds specific phosphotyrosine-containing proteins and peptides. We configured an FCS-based binding assay for this interaction using the soluble src-SH2 domain (M_r = 14 kDa) engineered to contain an N-terminal epitope tag for a high-affinity non-neutralizing antibody. Using a fluorescently-labelled phosphopeptide specifically recognized by src-SH2 (TMR-Ahx_2-pY; M_r = 2.5 kDa), we observed a 3.7-fold increase in diffusion time upon formation of the src-SH2–TMR-Ahx_2-pY binary complex (t_{free} = 120 μs; t_{bound} = 440 μs; M_r = 16.5 kDa) by FCS. This increase in diffusion time was amplified further ($t_{complex}$ = 550 μs) when the mass of the fluorescent src-SH2–TMR-Ahx_2-pY complex was enlarged by complexing SH2 with the non-neutralizing monoclonal antibody (mAb) that recognizes its epitope tag (M_r = 160 kDa, Fig. 2a). The K_d for TMR-Ahx_2-pY binding to either src-SH2–mAb (215 ± 18 nM, Fig. 2b) or src-SH2 alone (240 nM) is comparable with that obtained from competition studies in a conventional ELISA assay (200 nM) and also, using the biotinylated peptide, in competition FCS assays (K_i = 420 ± 45 nM, Fig. 2c) and in radioligand binding/ELISA assays (470 nM). The FCS assay volume was successfully reduced to 1.2 μl with no loss in performance (standard deviations = 5%, 1.6 s read times) and yielded identical K_d values to those obtained in 200 μl. Dose–response curves of unlabelled peptide and known small-molecule inhibitors randomized within 40 assay test plates were successfully identified in a test screen performed at Evotec Biosystems.

Peptide cleavage-based protease assay

Proteases are implicated in a wide range of pathologies and, as such, are relatively common targets for pharmaceutical intervention. We chose the serine protease essential for human cytomegalovirus replication (CMV-protease) to exemplify the development of FCS-based assays for proteolytic activity. A peptide substrate was prepared with biotin and Rhodamine Green (RhGn) on either side of the known CMV-protease cleavage site. Using this substrate, proteolysis could be readily monitored by FCS following addition of avidin, as the diffusion time of the substrate–avidin complex (t_b = 240 μs) differs from that of the RhGn-labelled cleavage product (t_f = 92 μs). Comparable k_{cat}/K_m values for cleavage of this substrate by CMV-protease were obtained using FCS (k_{cat}/K_m = 320 M^{-1} s^{-1}),

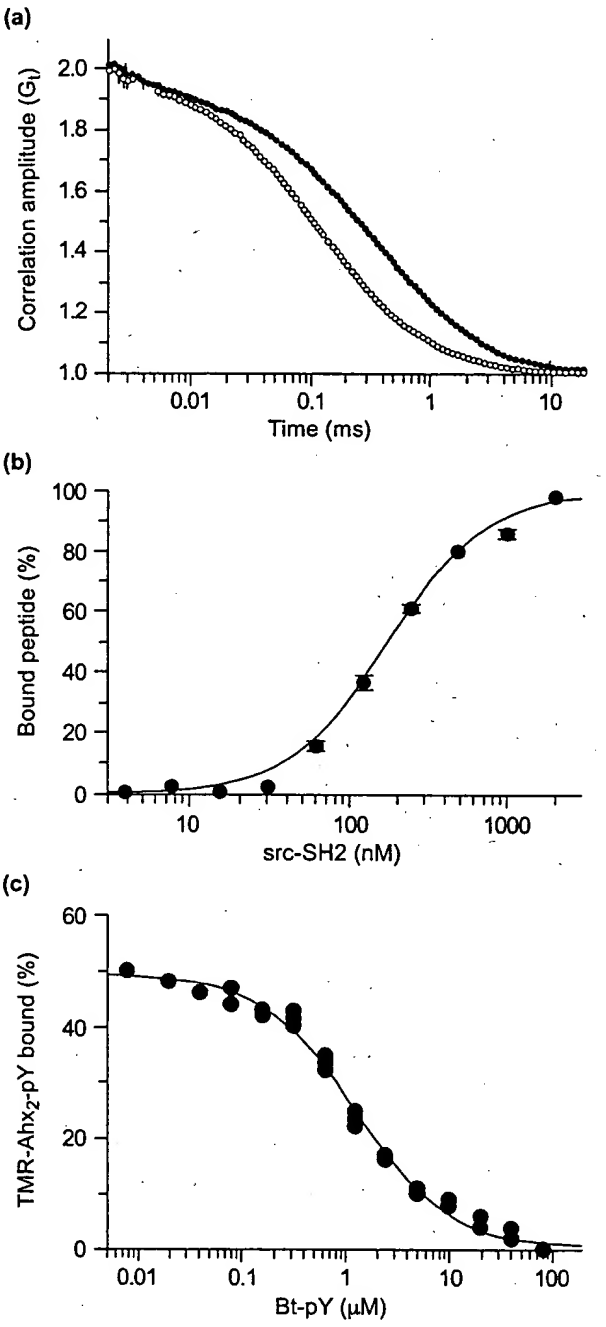

Figure 2. *FCS assay for peptide binding to src-SH2 domain: (a) correlation curves of free (white circle) and bound (black circles) fluorescently-labelled phosphopeptide, TMR-Ahx$_2$-pY, (b) TMR-Ahx$_2$-pY binding isotherm to src-SH2. K_d = 215 nM. (c) Displacement of TMR-Ahx$_2$-pY with biotinylated phosphopeptide, Bt-pY (K_i = 420 ± 45 nM). Error bars show SD.*

fluorescence anisotropy (k_{cat}/K_m = 300 $M^{-1}s^{-1}$) and time-resolved fluorescence (k_{cat}/K_m = 290 $M^{-1}s^{-1}$). In fact, from other examples of human, viral and bacterial proteases, near identical k_{cat}/K_m values were always obtained using FCS, anisotropy and HTRF approaches similar to that outlined above for CMV-protease. However, both anisotropy and HTRF assays required assay volumes of >10 μl to generate useful signals for test compound screening, compared to 1 μl or less for FCS.

FCS-based 7-TM receptor binding assays

G-protein-coupled (or 7-transmembrane; 7-TM) receptors represent possibly the single most important class of targets for current and prospective drug therapies. To date, few successful examples of the development of miniaturized homogeneous assays for ligand binding to these receptors have been reported. In this regard, FCS may provide a more-or-less unique solution. We have successfully configured membrane vesicle ligand-binding assays for all of the 7-TM receptors investigated to date (at expression levels of <100,000 receptors $cell^{-1}$). In one case, a peptide ligand 7-TM receptor, the binding and displacement of a TMR-labelled ligand could be monitored by FCS with K_d values (3 nM) comparable with those of the radiolabelled peptide (1.5 nM) and with <10% nonspecific binding. The binding of a TMR-labelled chemokine to its receptor also yielded K_d values (1.1 ± 0.3 nM) consistent with those obtained by scintillation proximity assay (0.8 ± 0.5 nM) and, again, with <10% nonspecific binding (Fig. 3). We have successfully measured specific ligand binding and displacement in this system with receptor expression levels <10,000 receptors per cell or in membrane preparations with a B_{max} ~0.2 pmol mg^{-1} measured by radioligand binding.

Binding of the human type-2 high-affinity somatostatin (SMS) receptor (sst_2) to its 7-TM receptor represents a further example of a successful vesicle-based FCS assay. Binding of a tetramethyl-rhodamine-labelled somatostatin-14 conjugate to small membrane vesicles prepared from CCL39 hsst2 cells overexpressing the receptor and competition with a non-labelled somatostatin-14 resulted in a K_d of ~1.6 nM for the natural ligand. With the application of specific assay conditions minimizing the surface adhesion and nonspecific binding of the labelled SMS, the miniaturization from the microscale to the nanoscale was possible without further optimization. Figure 4 shows a typical competition curve for a synthetic SMS analogue at fixed

nanomolar concentrations of 5TMR-SMS14 and sst_2-receptor-expressing vesicles. Together, these represent the first examples of the routine development of homogeneous fluorescent ligand-binding assays for this class of target in a miniaturized (<10 μl) form suitable for HTS screening, and as such, represent an important advance.

CD45 phosphatase – from assay development to prototype HTS screen

CD45 phosphatase, a transmembrane protein tyrosine phosphatase, is expressed in haematopoietic cells. It is one of the most abundant leukocyte cell-surface glycoproteins (Leukocyte Common Antigen, LCA). CD45 plays a pivotal role in antigen-stimulated proliferation of T cells. Two Src-family protein tyrosine kinases (PTKs), Lck and Fyn have been implicated as physiological substrates of CD45. The enzyme dephosphorylates the negative regulatory tyrosine residue of Lck, thereby serving as an obligate positive regulator.

The development of low molecular weight compounds that inhibit protein tyrosine phosphatase (PTP) activity of CD45 would potentially lead to novel drugs for suppression of immune and inflammatory reactions. The cytosolic part of the CD45 receptor consists of two tandem domains PTP-D1 and -D2. The catalytic activity is present in D1. With recombinant CD45 D1–D2, an ELISA assay had been established in the conventional 96-well plate format using biotinylated phosphotyrosine peptide substrates with different motifs for a specificity control-based *in vitro* screening program. After the enzymatic (inhibition) reaction was performed in homogeneous solution, the N-terminally biotinylated peptide recognition motifs were captured on a streptavidin microtiter plate to enable the detection reaction by a mouse-anti-phosphotyrosine/anti-mouse horseradish peroxidase-antibody cascade. As shown in Fig. 5, this solution ELISA assay format enabled a very quick adaptation to FCS and also to fluorescence anisotropy.

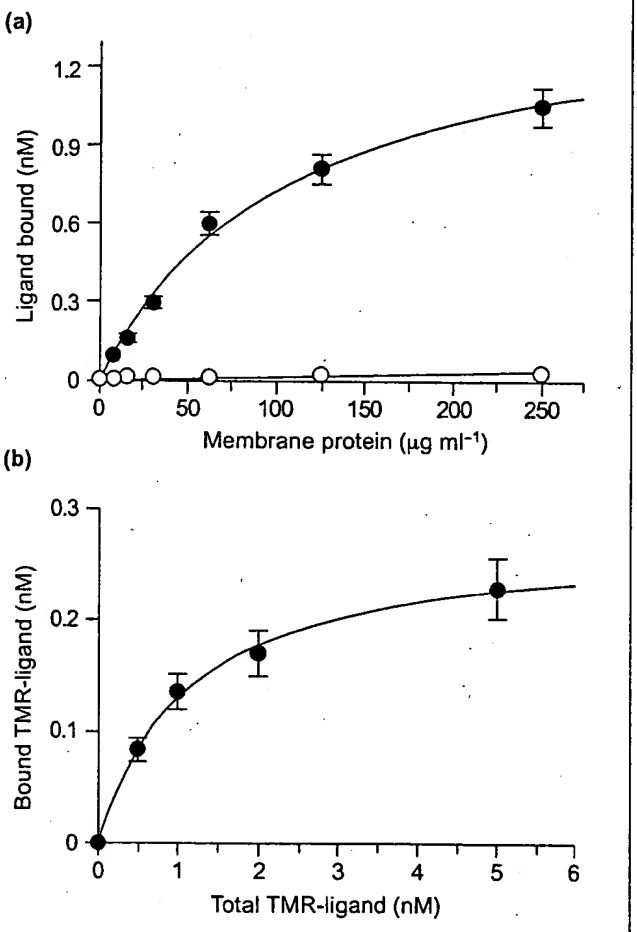

Figure 3. *Binding of TMR-labelled chemokine to membrane vesicles prepared from cells overexpressing the chemokine receptor. (a) Titration curve at fixed TMR–ligand concentration (1.5 nM) ± 1 μM unlabelled chemokine. Black circles, total binding, white circles, nonspecific binding. (b) Titration curve of TMR–ligand at 25 ng μl⁻¹ membrane protein, K_d = 1.1 ± 0.3 nM. Error bars show SD.*

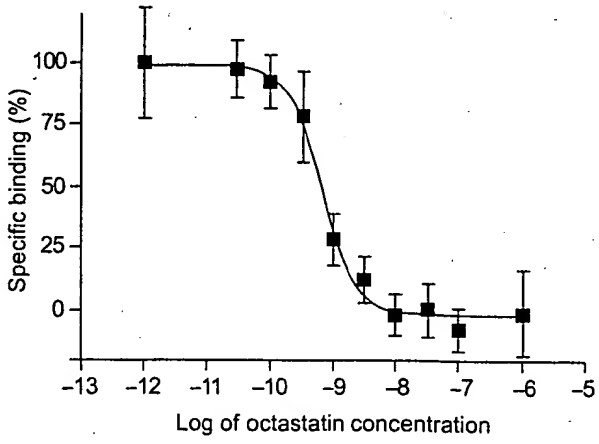

Figure 4. *SMS competition assay showing the dose–response curve for octastatin. Error bars show SD.*

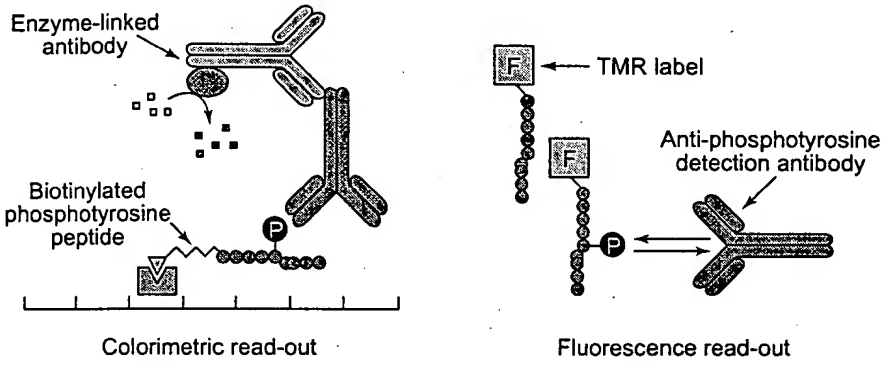

Figure 5. *CD45 phosphatase assay schemes. Left: solution mixing ELISA format. Right: homogeneous solution assay for fluorescence anisotropy and for translational diffusion measurements in FCS. F, fluorescent label; P, phosphate.*

Adaptation to FCS

The N-terminal-$(CH_2)_6$-linked biotin in the phosphotyrosine-containing peptide substrate was replaced by a TMR fluorescence label. The labelled substrate allowed for a very simple assay principle based on fluorescence anisotropy or FCS. Thus, in the absence of CD45 phosphatase activity or in the presence of an inhibitor the anti-phosphotyrosine detection antibody on binding to the peptide leads to a significant increase in rotational or translational diffusion time of the complex. If full cleavage of the phosphate occurs the detection antibody cannot bind and the anisotropy or FCS signal remains unaltered. Figure 6a shows a typical antibody equilibrium binding curve (K_d = 123 ± 15 nM) from a fluorescence anisotropy titration. The fluorescence label in the anisotropy experiment shown was EDANS. Figure 6b shows typical normalized autocorrelation curves of the free and Ab-bound TMR-(PO_3^{2-})–Y-peptide; it demonstrates the shift in translational diffusion time in relation to the data quality achieved in the measurement at 5 nM substrate concentration.

Systematic evaluation of the assay conditions in micro-format with specific emphasis on the following: ease of handling, robustness, sensitivity, reproducibility (different degrees of Ab binding and phosphatase reaction), order of addition of assay components, stop reagents, impact of organic solvent, incubation time and measurement time. The nano-format HTS run was then performed as follows:

Some 4000 pure organic substances and 4000 natural broths were screened by transferring 105 nl of each into prototype 200-well nanotiter plates. From a four-channel piezo-driven nanodispenser the assay components were transferred into the nanowells to yield a final volume of 1.3 µl per well. After preincubation of the CD45-LCA enzyme (150 nM) with the potential inhibitor to establish

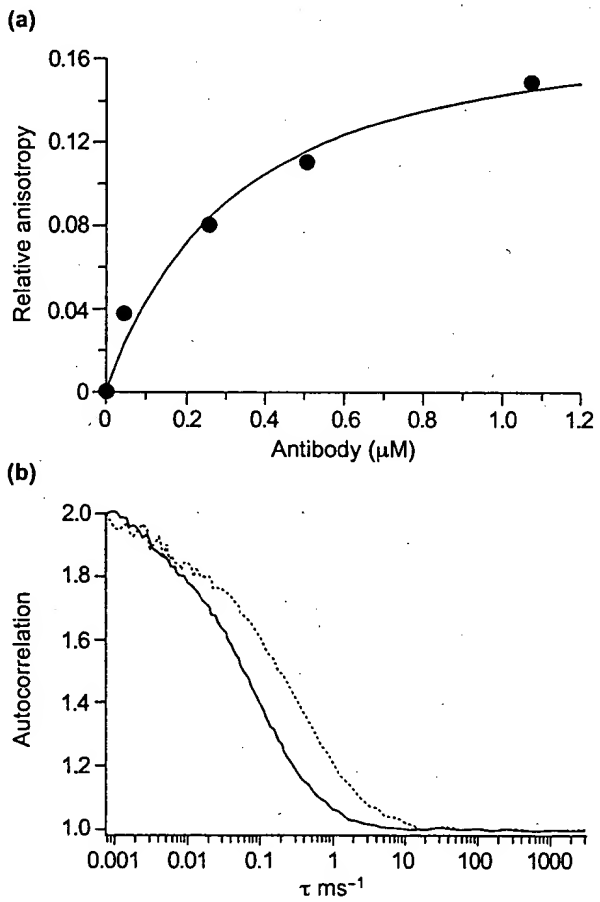

Figure 6. *(a) Fluorescence anisotropy equilibrium titration of EDANS-(PO_3^{2-})–Y-peptide with antiphosphotyrosine antibody (Ab). (b) Normalized autocorrelation curves for free TMR-(PO_3^{2-})–Y-peptide (solid line, τ = 128 µs) and the TMR-(PO_3^{2-})–Y-peptide–Ab complex (dashed line, τ = 488 µs).*

equilibrium, the TMR-peptide was added to a final concentration of 26 nM. After 20 min reaction time the detection antibody (2.5 μM) – stop reagent – was added as a premixed solution. Translational diffusion measurements followed with 1.6 s scanning time per well.

Practical assessment

This non-automated demonstration screening run resulted in 10,332 assays performed in 46 h. (More than 12 million individual droplets of ~1 nl were dispensed, corresponding to an average drop frequency of 70 drops s^{-1}). The test run was performed in a double-blind study with two times five positive compounds spiked into the 8000 samples that were run in parallel in the ELISA assay format. Inhibitors were identified in the initial screening run from the percentage of inhibition of the CD45 enzymatic activity. One of the major advantages of FCS-based HTS is the possibility for parallel biophysical characterization of positive hits in the primary screening run. No other screening technique described so far allows the immediate mechanistic characterization of inhibitors by determination of highly accurate kinetic and thermodynamic data at the same quality level as FCS. After confirmation of a positive hit by measurement of dose–response curves (see Fig. 7 for a typical example of a dose–response curve from a positive hit in the CD45 assay), compounds which are still found to be active can be very efficiently characterized by determination of their kinetic and thermodynamic interaction and/or inhibition parameters.

One important parameter in enzymatic assays is the association rate between inhibitor and enzyme. If the ligand/competitor association is much slower than the kinetics of the enzymatic reaction, the test compounds have to be preincubated with the enzyme to separate association and substrate cleavage. A plot of the inverse time constants of the association reaction versus the total inhibitor concentration under pseudo first-order conditions provides the association rate in accordance with $1/\tau = k_{+i}[I]_0 + k_{-i}$ (Fig. 8).

Single-turnover evaluation

In classical steady-state enzyme kinetics the time-consuming approach to derive physical constants (k_{cat}, K_m) from enzymatic reactions is using the Michaelis–Menten model. The prerequisite for this formalism is that the substrate concentration must be much higher than the enzyme concentration such that the substrate concentration effectively remains

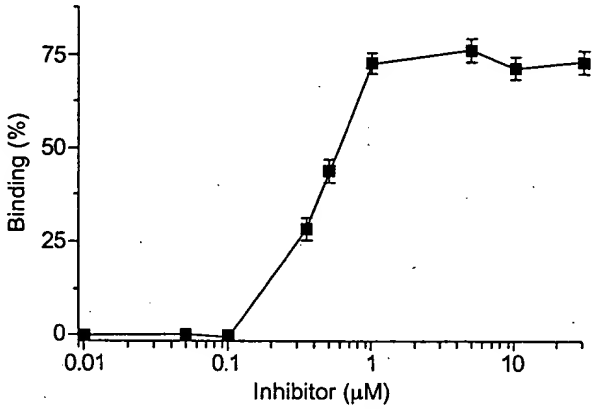

Figure 7. *Determination of a typical dose–response curve of a CD45 phosphatase positive hit found in the initial HTS.*

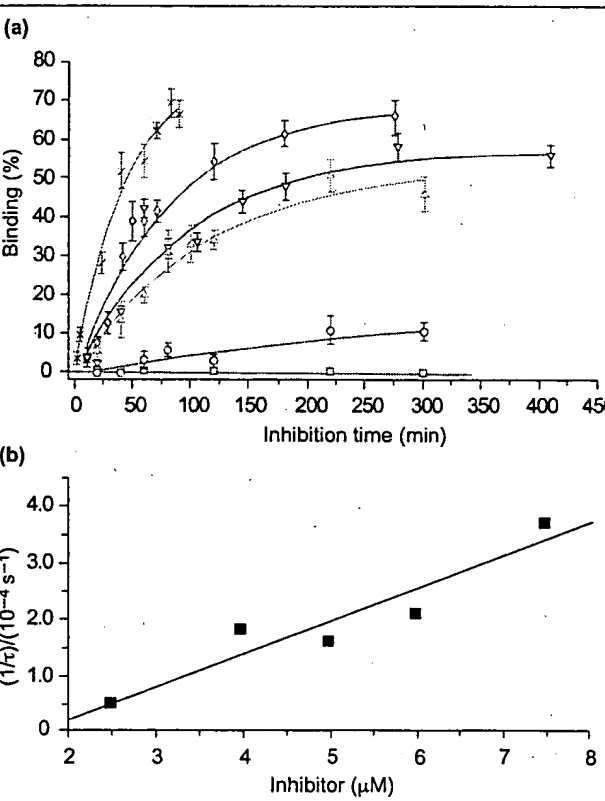

Figure 8. *(a) Association kinetics of 10 nM CD45–LCA at different concentrations of inhibitor: red, 1 μM; green, 2.5 μM; light blue, 4 μM; pink, 5 μM; dark blue, 6 μM and orange, 7.5 μM; error bars show SD. (b) Determination of apparent pseudo first-order association rate of an exemplaric inhibitor;* $k_{on} = 59\ M^{-1}\ s^{-1}$.

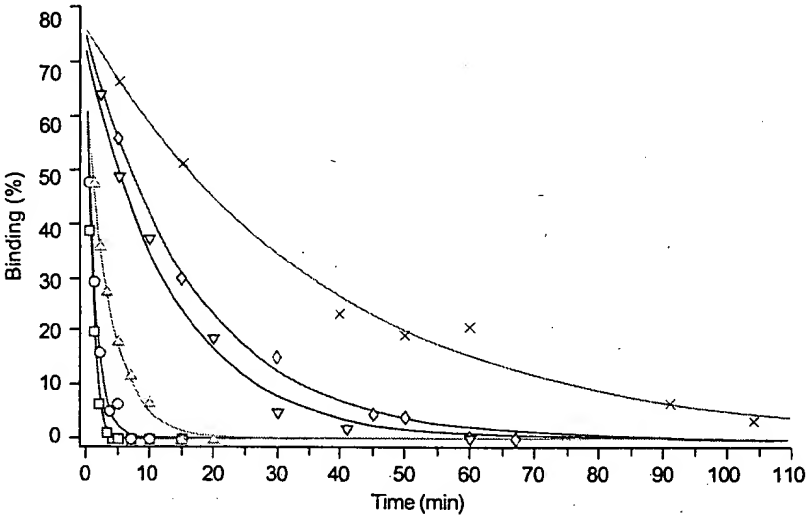

Figure 9. *Dephosphorylation kinetics of CD45-LCA in the presence of different concentrations of inhibitor: red, blank, t = 0.9 min; green, 1 μM, t = 1.5 min; light blue, 2 μM , t = 4 min; pink, 3 μM , t = 13.7 min; dark blue, 3.5 μM, t = 17.1 min and orange, 4 μM, t = 38.1 min.*

constant. Typically, FCS experiments are performed under single-turnover conditions. High substrate concentrations are disadvantageous because of aggregation phenomena and low solubility of substrate. The CD45-LCA assay described above clearly cannot be evaluated according to the general steady-state Michaelis–Menten approach. Therefore, a formalism was derived that allows for the determination of K_i values from the ratio of the time constants of the enzyme reaction with and without inhibitor (Fig. 9).

This formalism can be applied to all enzyme reactions where the turnover of small substrate concentrations is to be measured. It is particularly useful for FCS assays which, because of very high sensitivity and selectivity, allow enzymatic assays to be performed at low substrate concentrations.

Summary

The FCS assays we have developed thus far include examples of protein–DNA (Fig. 1), protein–peptide (Fig. 2), protein–protein, DNA–DNA, ligand–RNA, protein–RNA, protease, phosphatase (Figs 5–9) and 7-TM receptor targets (Figs 3,4). Experiments to investigate the utility of FCS to monitor cell surface and intracellular processes in living cells are ongoing. It is envisaged that hit profiling will be possible in the native cellular context using the same FCS assay used for the *in vitro* HTS screen. We expect that the power of FCS as a versatile *in vitro*, and ultimately intracellular, screening tool will become apparent as the industry-wide drive towards miniaturization approaches in assay volumes of 1 μl and below continues. The only limitation to homogeneous assays and confocal fluorescence detection is a maximal well concentration of fluorescent ligand of 1 μM, although, most FCS (and anisotropy) experiments are performed with nM concentrations of fluorescent ligand and the binding component (e.g. receptor) in molar excess, governed by the affinity of the interaction. Nevertheless, when the ligand is used in excess, 1 μM is well above biologically relevant levels. Finally, novel data processing algorithms developed at Evotec have removed the requirement for a mass change associated with the interaction of interest and the development of a parrallel FCS reader is expected to increase throughput by tenfold compared with the instrumentation used here.

ACKNOWLEDGEMENTS

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New assay technologies for high-throughput screening

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The use of high-throughput screening for early stage drug discovery imposes several constraints on the format of assays for therapeutic targets of interest. Homogeneous cell-free assays based on energy transfer, fluorescence polarization spectroscopy or fluorescence correlation spectroscopy provide the sensitivity, ease, speed and resistance to interference from test compounds needed to function in a high-throughput screening mode. Similarly, novel cell-based assays are now being adapted for high-throughput screening, providing for *in situ* analysis of a variety of biological targets. Finally, recent advances in assay miniaturization mark a transition to ultra high-throughput screening, ensuring that identification of lead compounds will not be the rate-limiting step in finding new drugs.

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Abbreviations

CRE	cAMP response element
FCS	fluorescence correlation spectroscopy
GFP	green fluorescent protein
HTS	high-throughput screening

Introduction

Continuing advances in molecular biology, human genetics and genomics have accelerated identification of the mechanisms underlying a growing number of human diseases. This progress has increased the number of novel protein targets available for potential therapeutic intervention by drug treatment. Concurrently, novel approaches in combinatorial chemistry and expanded collections of natural products have dramatically increased the number of compounds that can be tested for activity against these targets. The confluence of these two trends towards more potential targets and larger chemical libraries has greatly stimulated adoption of high-throughput screening (HTS) as the primary tool for early stage drug discovery.

HTS is the process by which large numbers of compounds are tested, in an automated fashion, for activity as inhibitors or activators of a particular biological target, such as a cell surface receptor or a metabolic enzyme. Although any assay performed on the bench top can, in theory, be applied in HTS, conversion to an automated format imposes certain constraints that affect the design of the assay in practice. Procedures that are routine at the bench are often extremely difficult to automate. Also, the more steps required for an assay, the more difficult to automate the HTS. The ideal assay is one that can be performed in a single well with no other manipulation other than addition of the sample to be tested.

A number of assay formats have been developed or modified over the past few years to conform to the constraints imposed by HTS. These assay protocols can be divided into two groups: cell-free assays that measure the biological activity of a relatively pure protein target and cell-based assays that assess the activity of a target, protein by monitoring a biological response of a cell in which the target protein resides. In either case, the protocols require minimal manipulations, can be performed robotically in relatively small volumes, yield robust responses and are relatively impervious to perturbation by solvents and compounds used in drug screening. In this review we describe several of the more recently developed or exploited assay protocols for HTS.

Cell-free assays

The primary goal in adapting cell-free assays to HTS is to minimize the number of steps required in setting up the assay and in detecting the activity, be it an enzymatic reaction or the binding of two components. This goal has been met to a large extent by development of detection systems that do not require separation of the product of the reaction from substrate, or from other components of the assay mixture. Earlier approaches to such homogeneous assay formats relied on proximity-dependent energy transfer. The output of such assays derived from the signal enhancement generated by bringing a source and a distance-dependent amplifier close together. For example, the β-particles of a low-energy radionuclide attached to a ligand will stimulate the fluorescent emission of a scintillant in a bead to which the ligand's receptor is attached [1,2]. More recently, this detection method has been applied to enzymatic reactions, such as that catalyzed by topoisomerase I [3]. As another example of energy transfer assay formats, the rare earth metal lanthanide, Eu^{2+}, when irradiated by light, can transfer its excitation energy in a nonradiative process to the fluorescent protein, allophycocyanin, if the two are in close proximity. This can occur when a Eu^{2+}-derivitized ligand binds to an allophycocyanin-linked receptor [4,5] or a Eu^{2+}-derivitized anti-phosphotyrosine antibody binds to a detector-linked phosphorylated substrate of a tyrosine kinase such as src [6•]. Use of time resolved fluorescent procedures assessing emission at specific times following excitation enhances the sensitivity of this technique by reducing interference from background fluorescence, from test compounds or from assay components [6•,7•]. Finally, enzymatic assays suitable for HTS and based on fluorescent resonant energy

transfer between two different forms of green fluorescent protein (GFP) have recently been described [8•].

A number of investigators have exploited fluorescence polarization spectroscopy (FPS) as the basis for homogeneous HTS assays of both enzymatic and binding reactions. When fluorescent molecules in solution are excited with polarized light, the degree to which the emitted light retains polarization depends on the extent to which the fluorescent molecule rotates during the interval between excitation and emission. The rapid rotation of small fluorescent molecules in solution results in substantial loss of polarization. If such small molecules bind to larger molecules, their rotational diffusion is reduced and the retention of polarization is correspondingly increased. Thus, by measuring the relative intensity of emitted light in the planes normal and orthogonal to the plane of the incident polarized light, the extent of rotation of a target molecule, and inferentially, the extent of binding of the target molecule to a larger component, can be calculated. For instance, fluorescent polarization has been used to detect the presence of specific drugs or hormones [9,10], to assess antibody binding of fluorescein-conjugated peptides [11] or to monitor DNA:DNA hybrid formation [12]. The recent availability of a 96-well plate reader [13] with a high sensitivity to fluorescein and fluorescein conjugates has allowed development of 96-well based fluorescent polarization assays. Such high-throughput assays for src family tyrosine kinase activity [14•], for binding of phosphopeptides to Src SH2 domains [15•], for interaction between STAT1 and an γ-interferon receptor-derived phosphotyrosine-containing peptide [16•] and for specific protease activities [17,18•] have recently been described. The sensitivity of fluorescence polarization, the ease and speed with which such assays can be run and the resistance of such assays to interference from absorptive compounds commonly present in complex mixtures [18•] make this procedure highly amenable to HTS.

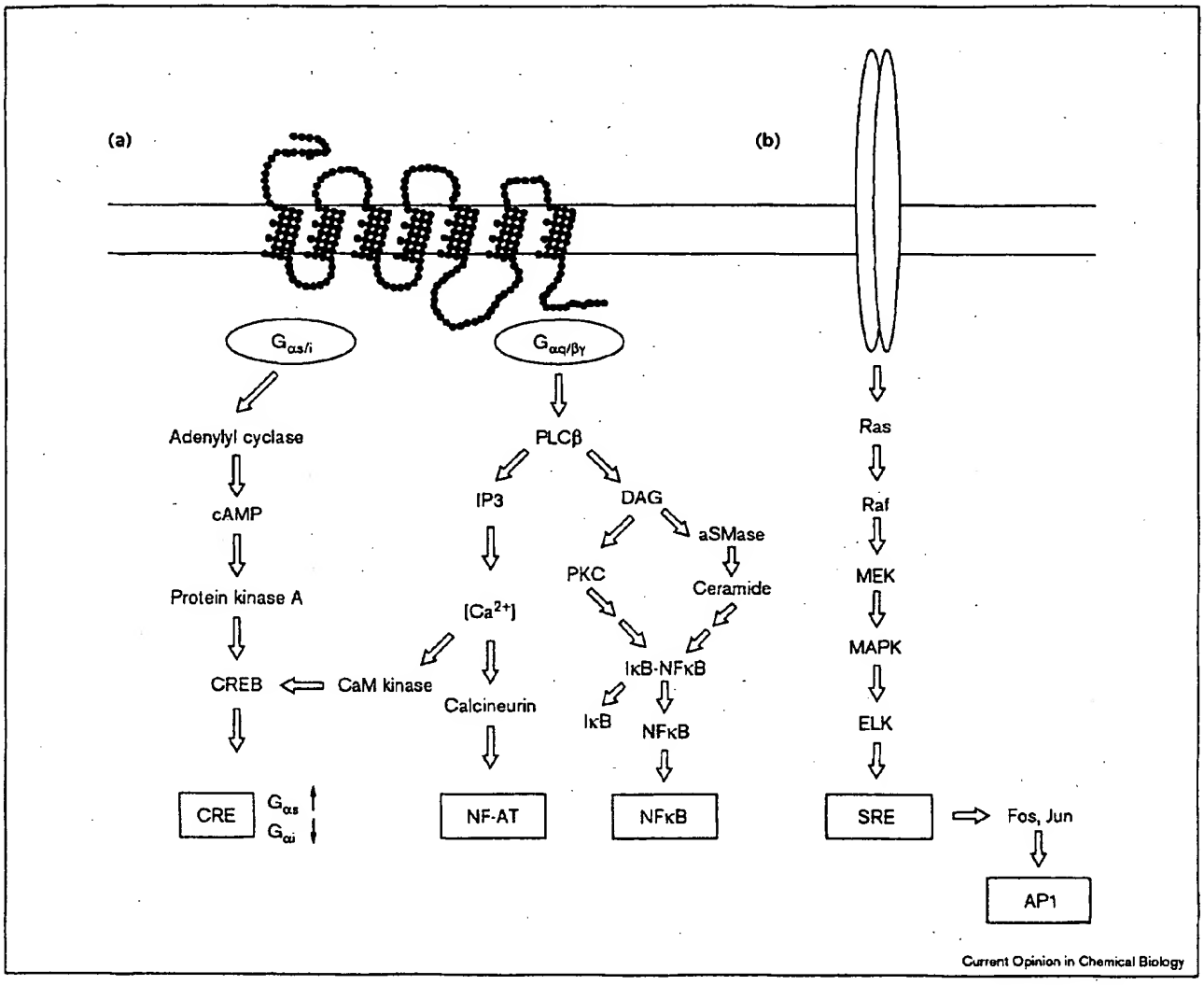
Fluorescence correlation spectroscopy (FCS) represents another recently developed detection format eminently suitable for HTS. FCS measures differences in physical states of a target molecule, such as bound versus free or cleaved versus intact, in a homogeneous mixture [19]. Specifically, FCS measures the burst of fluorescent emission of a molecule passing through a small volume of space, which is defined by a sharply focused laser beam. Small molecules diffuse through the volume rapidly and thus yield short bursts of light. Binding of these small molecules to larger molecules reduces their translational diffusion and correspondingly increases the duration of the bursts of light. Deconvolution of the emission patterns in a sample by appropriate software can yield the relative amount of the bound and unbound states of a fluorescently tagged ligand. This technology can therefore readily be applied to measure receptor–ligand interactions, DNA–protein interactions, nucleic acid hybrid formation and certain enzymatic reactions [20].

Cell-based assays

Cell-based assays are an increasingly attractive alternative to *in vitro* biochemical assays for HTS. Such *in vivo* assays require an ability to examine a specific cellular process and a means to measure its output. For instance, agonist activation of a cell surface receptor or a ligand-gated ion channel can elicit a change in the transcription pattern of a number of genes. This ligand-induced alteration in transcription can be readily captured by using gene fusions, in which a promoter element responsive to receptor activation is fused to the coding region for an enzyme or protein whose levels can be easily measured. Appreciation of the particular signaling pathway associated with a specific receptor allows identification of the appropriate transcriptional response element required to detect a response. Figure 1 depicts a number of signal transduction pathways, indicating the transcriptional response elements coupled to each pathway. Several reporter genes that generate products that can be adapted to HTS format are available [21,22]. These are listed in Table 1, with references to recent innovations in their use [23•,24,25,26•]. For instance, the recent report of novel fluorescent, cell-permeable substrates for β-lactamase documents the use of β-lactamase to detect receptor activation in single cells, making it an attractive assay system for high density HTS [27••].

While cell-based assays using reporter genes have proved effective as an HTS format, detecting more immediate responses to target protein activation provides several advantages, including shorter duration of the assay and fewer false positives from nonspecific interactions. As indicated in Figure 1, such cellular response dependent on activation of a receptor include elevation of a second messenger (for example, Ca^{2+}, cAMP, inositol triphosphate), phosphorylation of an intermediate signaling protein, or subcellular translocation of a signaling molecule. Recent advances in molecular biology and in instrumentation have made it possible to monitor these events in an automated format. For instance, the recent availability of a 96-well fluorescent imaging plate reader (Molecular Devices, Sunnyvale, California, USA) permits HTS of receptor activation by monitoring Ca^{2+} mobilization of cells preloaded with a fluorescent calcium indicator, such as FLUO-3 (Molecular Probes, Eugene, Oregon, USA). In addition, recombinant cells expressing a calcium-sensitive fluorescent protein, such as aequorin [28•] or a hybrid calmodulin–GFP protein [29••], obviate the need for preloading cells with dyes in order to detect calcium fluxes following stimulation. A separate approach to detecting early events following receptor stimulation involves examining relocalization of specific components of the signal transduction machinery. For instance, MAP kinase (Figure 1) relocalizes from the cytoplasm to the nucleus within minutes following stimulation of an upstream G-protein-coupled receptor [30,31]. Similarly, Barak *et al.* [32•] have shown that recruitment of a β-arrestin–GFP fusion protein to the plasma membrane can be used to monitor activation

Figure 1



Signal transduction pathways commonly used in mammalian cell-based high-throughput assays. **(a)** Agonist-engaged seven transmembrane receptors are functionally linked to the modulation of several well characterized enhancer/promoter elements, the cAMP response element (CRE), nuclear factor of activated T cells (NF-AT), NFκB, serum response element (SRE) and AP1 [46–49]. Upon activation of a $G_{\alpha s}$ coupling receptor, adenylyl cyclase is stimulated, producing increased concentrations of intracellular cAMP, stimulation of protein kinase A, phosphorylation of the CRE binding protein (CREB) and induction of promoters with CRE elements. $G_{\alpha i}$ coupling receptors dampen CRE activity by inhibition of the same signal transduction components. $G_{\alpha q}$ coupling receptors and some βγ pairs stimulate phospholipase C (PLC), and the generation of inositol trisphosphate (IP3) and diacylglycerol (DAG). A transient flux in intracellular calcium promotes induction of calcineurin and NF-AT, as well as calmodulin (CaM)-dependent kinase and CREB. Increased DAG concentrations stimulate protein kinase C (PKC) and endosymal/lysosomal acidic sphingomyelinase (aSMase); while the aSMase pathway is dominant, both induce degradation of the NFκB inhibitor IκB as well as NFκB activation. By a poorly understood mechanism, IκB degradation may also be initiated through the MAPK (mitogen-activated protein kinase) cascade (not shown). **(b)** Growth factor receptor (depicted by ellipses) activation results in recruitment of Sos (not shown) to the plasma membrane, where it stimulates Ras, which recruits the serine/threonine kinase Raf to the plasma membrane. Once activated, Raf phosphorylates MEK kinase, which phosphorylates and activates MAPK and the transcription factor ELK (Ets-like protein, also known as p62 TCF1 [ternary complex factor 1]). ELK drives transcription from promoters with SRE elements, leading to synthesis of the transcription factors Fos and Jun, that form a transcription complex capable of activating AP1 sites. Seven transmembrane receptors also stimulate the MAPK pathway through βγ subunits, most probably through phosphoinositide 3-kinase γ (PI3Kγ; not shown).

of a number of different G-protein-coupled receptors. Recent advances in microscopic imaging technology, in conjunction with software permitting automated image recognition, provide a means to capture these events in a high-throughput mode.

Cell-based assays have significant advantages over *in vitro* assays. First, the starting material (the cell) self-replicates, avoiding the investment involved in preparing a purified target, in chemically modifying the target to suit the screen, and so on. Second, the targets and readouts are ex-

Table 1

Reporter genes useful for cell-based high-throughput screening.

Reporter genes (source)	Advantages	Disadvantages	References
β-galactosidase (bacterial)	Well characterized; stable, inexpensive substrates; highly sensitive fluorescent or chemiluminescent substrates available; little interference from test compounds; simple readouts (readily automated)	Endogenous activity (mammalian cells); tetrameric (non-linear response at low concentration)	[23•,50]
Luciferase (firefly)	Dimeric; high specific activity; no endogenous activity (low background)	Requires addition of cofactor (luciferin) and presence of O_2 and ATP	[23•]
Alkaline phosphatase (human placental)	Secreted protein (avoids the need for membrane-permeable substrates); inexpensive colorimetric and highly sensitive luminescent assays available	Endogenous activity in some cell types; optimal at pH 9.8	[24,25]
β-lactamase (bacterial)	Monomeric; highly sensitive fluorogenic substrates described; no endogenous activity	Membrane-permeable fluorescent substrates not readily available	[27••]
GFP (jellyfish)	Monomeric; no substrate needed (no manipulations required for assay); no endogenous activity; multiple forms available	Relatively low specific activity	[26•,51,52]

amined in a biological context that more faithfully mimics the normal physiological situation. Third, cell-based assays can provide insights into bioavailability and cytotoxicity. Mammalian cells are expensive to culture and difficult to propagate in the automated systems used for HTS, however.

An alternative to mammalian cell based assays is to recapitulate the desired human physiological process in a micro-organism such as yeast [33]. For instance, signaling via human G-protein-coupled receptors has been reconstituted in yeast to yield a facile growth response or a reporter gene readout ([34,35]; Klein *et al.*, unpublished data). Similarly, mammalian ion channels have been coupled to growth response in yeast [36]. Also, protein–protein interactions, including RAS–RAF association [37] and tyrosine kinase receptor–ligand binding [38], have been faithfully reproduced using the yeast two-hybrid system. Finally, many mammalian transcription factors operate in yeast, including glucocorticoid receptor [39,40] and the retinoic acid receptor and retinoid X receptor families of receptors [41]. The ease and low cost of growing yeast, their ready genetic manipulation, and their resistance to solvents make yeast an attractive option for cell-based HTS.

Miniaturization

Several factors are fueling efforts to increase the speed of HTS and decrease the volume of individual reactions within an HTS format. Split-bead synthesis (see Note added in proof), or other similar approaches to combinatorial chemistry, dramatically increases the number of compounds that can be produced in a library but do so at the cost of quantity of material. In addition, the limited supply of existing compounds within chemical libraries of pharmaceutical companies, and the growing number of targets against which such compounds can be tested, motivate a frugal approach to use of those compounds. Finally, the reagent costs associated with HTS, when multiplied by the increasing number of assays per run, are becoming a significant cost of early stage drug discovery.

In response to these exigencies, a number of groups have begun to develop formats for very high density screening using very small assay volumes. One approach involves reducing the well size and increasing the density of the assay plate but retaining the overall assay format used in current 96-well based HTS. Densities of 6500 assays in a 10 cm array have been reported for cell-free enzyme based assays [42•] and for ligand binding in cell based assays [43••]. This approach of miniaturizing existing formats significantly increases the number of assays per plate and the overall throughput of the screen but is intrinsically limited by the physical constraints of delivering small volumes to wells, and of detecting responses in a sensitive and timely manner. Accordingly, novel formats have been developed that eschew the assay format based on wells. One approach uses glass chips containing microchannels in which reagents, target proteins and compounds are herded by electrokinetic flow controlled by electric potentials applied at the ends of the channels [44•]. A related approach attains high-throughput both of chemical synthesis and activity assessment by parallel arrays of three-dimensional channels in which flow is controlled by miniature hydrostatic actuators [45]. These approaches provide significant reduction in the volume of assays and a corresponding savings in reagent costs over conventional HTS [45]. In addition, with further development in parallel processing in multiple chips, the number of assays performed in a given period

of time can increase dramatically. This movement to miniaturization is likely to ensure that the initial stage of drug discovery identification of lead compounds will not be the rate-limiting step in finding new drugs.

Conclusions

The last decade has witnessed the emergence across the pharmaceutical industry of the 96-well-based, robotics-driven, high-throughput screening process as the primary tool for identifying active compounds in the first stage of drug discovery. This program has dictated the format of the assays that are used to assess the activities of targets — enzymes, receptors, transporters and so on — that underlie drug discovery in various therapeutic areas. A number of such formats — resonant energy transfer and fluorescent polarization spectroscopy in cell-based assays — have gained widespread acceptance and growing incorporation into high-throughput screening programs. The growing number of potential therapeutic targets, the increasing number of screenable compounds, the accelerating costs of screening and the increasing pressure to generate more lead compounds in a shorter time all conspire to render even the new approaches inadequate for meeting the anticipated throughput requirements, however. Thus, we are likely to witness a movement towards even greater screening throughput by miniaturization and increased reliance on robotics. Whether a new standard format for screening emerges in the near future, or whether a variety of formats are pursued concurrently remains to be seen. Nonetheless, we can anticipate that the exigencies of drug screening will motivate a continued application of state-of-the-art technologies to the process of high-throughput screening.

Note added in proof

For a reference describing split-bead synthesis, see [53].

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